

**IDENTIFICATION AND CHARACTERIZATION
OF AN ONCOGENICALLY MODIFIED
ACTIN ASSOCIATED PROTEIN**

by

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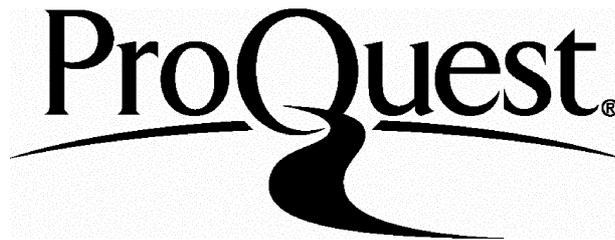
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ABSTRACT

The actin cytoskeleton is crucial for a variety of cellular events such as cell locomotion, phagocytosis, cytokinesis and cell surface receptor movement. The control mechanisms for these actin based cellular events are provided by a large number of actin-associated proteins which, acting in concert, regulate the polymerization status, interactions and geometry of actin. I have characterized a new actin associated 21 kDa polypeptide doublet, protein C4. In normal mesenchymal cells, protein C4 is associated with and uniformly distributed on actin stress fibres, while in cells and tissues that lack stress fibres, C4^l is the only protein C4 isoform present. Protein C4 is present in all cells and tissues so far examined apart from neurones, erythrocytes and skeletal muscle. Protein C4 is evolutionarily conserved as it has been found in yeast.

A number of actin associated proteins are down regulated in transformed cells in parallel with the reorganization of the actin cytoskeleton that often accompanies transformation. I have shown that the higher Mr protein C4 isoform, transgelin, is absent in oncogenically transformed cells where actin stress fibres are reduced in number or absent, while in contrast, the lower Mr protein C4 isoform, C4^l, is always present. Expression of transgelin can also be blocked by culturing normal, non-transformed mesenchymal cells in suspension. Re-expression of transgelin occurs 24 hours after these cells are returned to normal adherent culture conditions, but can be blocked by either actinomycin D or cycloheximide, suggesting that the expression of transgelin is regulated at the transcriptional level.

I have purified transgelin and shown that it binds directly to actin filaments at a ratio of 1 : 6 actin monomers, with a binding constant (K_a) of $7.5 \times 10^5 M^{-1}$, and that it induces actin filament gelation within 2 minutes, without affecting actin polymerization. Although both actin binding and gelation activities of transgelin are controlled by ionic strength, and may be mediated by positively charged amino acid residues, the molecule remains as a monomer irrespective of ionic conditions. Electron microscopy reveals that transgelin converts actin filaments from a loose random distribution into tightly tangled aggregates. An 'add-back' permeabilization system shows that transgelin specifically rebinds to actin filaments in cells from which it has previously been removed by detergent extraction.

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INTRODUCTION

I

1. THE CYTOSKELETON

The cytoskeleton of eukaryotic cells is involved in a number of fundamental cellular processes, including cell motility (2), cell division, intracellular transport, the movement of cell surface receptors and the maintenance of cell shape and structural rigidity (25,369,89,5). The cytoskeleton is composed of three filamentous systems, microtubules (280), intermediate filaments (250) and microfilaments (248), which interact with each other to form a structure that functions in a coordinated manner (166,25,151,446).

An ever increasing number of proteins are found in association with all three filament networks, and they are involved in the regulation of network structure, functions and interactions (351,295,451,116).

The structure and function of microtubules, intermediate filaments and their associated proteins are briefly outlined below. Microfilaments, their associated proteins, functions and role in transformation are the subject of my thesis and will be discussed in greater detail.

2. MICROTUBULES

Microtubules are found in most cells and are the main structural element in, for example, the axoneme in eukaryotic cilia and flagella. Microtubules play an important role in cell division, forming a mitotic spindle in association with the centriole, that aligns and separates chromosomes in dividing cells (302), while in conjunction with intermediate filaments and actin microfilaments, they establish and maintain cell shape, and the overall structure, architecture and organization of the cytoplasm. Microtubules also support bidirectional organelle and vesicle transport, acting as tracks for motor proteins such as dynein and kinesins.(236)

In interphase cells microtubules radiate in a complex web from a microtubule organising centre (MTOC), near the nucleus. This region contains colchicine resistant microtubules, and nucleates new filament assembly. The MTOC contains the centriole, a pair of specialised microtubule assemblies, containing nine sets of three microtubules

perpendicular to each other, that replicates at the end of interphase and form the spindle poles of mitotic cells (5).

Microtubules are composed of α and β tubulin subunits, each 55 kDa, that exist as a heterodimer. Each subunit of a tubulin dimer has a binding site for a guanine nucleotide. While either GTP or GDP can bind to β tubulin and exchange with a free nucleotide after hydrolysis, only GTP can bind to α tubulin, where it is non exchangeable, non hydrolysable, and may serve a structural role (281). On polymerisation these dimers join head to tail to form protofilaments which then assemble into hollow cylindrical microtubules by lateral bonds between subunits of the protofilaments. Most native microtubules contain 13 protofilaments with a diameter of 25nm (280).

Most species have more than one gene for each tubulin monomer, and in higher eukaryotes there are 5 α genes and 6 β genes, with the expression of tubulin isotypes developmentally controlled, in a tissue and cell specific manner (280). Further complexity is introduced by post-translational modifications, including phosphorylation, which inhibits assembly, reversible detyrosination and acetylation of the α subunit, which alters polymer stability, and polyglutamylation (268).

A third type of tubulin subunit, which has 30% homology with the amino acid sequence of α or β tubulin has been identified. γ tubulin can form dimers with α or β subunits and is associated with microtubule organising centres (MTOC) from which interphase microtubule radiate. This isoform both nucleates microtubule assembly in MTOCs, and by specifically interacting with β tubulin, establishes the structural polarity of microtubules in the cell (268, 312).

Microtubules are dynamic structures *in vivo* and *in vitro*, and as they are polar structures, dimer addition and loss occurs at different rates at the + (fast) and the - (slow) end. *In vitro* the + end is more dynamic than the - end, reflecting the different properties of the two ends of the microtubule. *In vivo*, reactions are largely restricted to + end as the - end is anchored at a MTOC (301). Polymerisation of microtubules *in vitro* has an initial lag phase (that can be accelerated by the addition of microtubule fragments as seeds) followed by an elongation step, which is fastest with dimers that have two GTP molecules bound. Following addition to a growing microtubule, the β tubulin GTP hydrolyses to

GDPPi/GDP, which is thermodynamically poised to depolymerise, therefore the polymer containing it is destabilised and the microtubule shrinks unless stabilised by the continuous addition of subunits. As GTP hydrolysis lags behind addition, a stabilising GTP (or GDPPi) cap may exist at the end of a microtubule and promote further growth. Microtubules can switch between phases of growth and shrinkage, so that even when the population as a whole is at steady state, individual microtubules elongate and shrink. This phenomenon of dynamic instability has been observed *in vivo* and *in vitro*. (58, 60, 301, 376).

Many proteins are associated with microtubules, acting to control their assembly, disassembly and interactions. They can be classified into two groups, motor and non motor microtubule associated proteins (MAPs).

MAPs bind to the outer surface of microtubule, and promote their assembly by enhancing the association of subunits. By stabilising microtubule subunit interactions MAPs suppress dynamic instability, so that microtubules primarily grow. By promoting the formation of tubulin oligomers MAPs can induce tubulin assembly in low levels of GTP, because it is not necessary for all the tubulin subunits to have GTP bound for the oligomer complex to bind on to the microtubule end. MAPs also have a role in maintaining the ordered structure of microtubules, possibly by promoting the formation of normal lateral bonds between protofilaments so that in the absence of MAPs, these may assemble into aberrant forms such as twisted ribbons and curved sheets (254).

MAPs also act as cross linkers and either tau or MAP2 introduced into fibroblasts induces bundling of normally separate microtubules. In addition, MAP2 and MAP1A have been shown to be components of the fine filaments that connect the parallel microtubules in neuronal processes. MAPs can also bind to both microtubules and microfilaments, thus connecting these two cytoskeletal filament systems (254).

Other non motor MAPs have more specialised roles. For example, nexins hold together the outer doublet of microtubules in the axoneme and MAPs that initiate and organise microtubule assembly are found in the MTOC of basal bodies that lie at the base of flagella. Proteins that are associated with the kinetochore are responsible for the capturing ends of

microtubules during spindle formation are also capable of nucleating microtubule assembly (236).

Motor proteins

Microtubule associated motor proteins have Mg ATPase activity, that is stimulated by binding to microtubules, and they use this energy source to move unidirectionally along microtubules (389). For example, kinesins moves from the - to the + end of microtubules, and *in vivo* translocate organelles from the centre to the periphery of cells. While their head domains, which contain the microtubule and ATP binding site, are conserved, the tail domains are not, as they are adapted for binding to different types of cargo. A family of KRP proteins (kinesin related proteins) which vary in motor properties, for example, rate and polarity of microtubule movement, has also now been identified.

Another group of motor proteins, microtubule associated cytoplasmic dyneins support retrograde movement of organelles. An inactive form is transported to the cell periphery before it directs transport of membrane bound particles to the - end of a microtubule. A bidirectional, or two very closely related forms of dynein has been identified. A further role for dynein has been suggested by the finding that it may act as a mitotic motor, directing the poleward movement of chromosomes along microtubules in mitosis (450, 382, 408, 138).

3. INTERMEDIATE FILAMENTS

Intermediate filaments are so called because their diameter of 10nm lies between that of microfilaments and microtubules (141). They form an intricate filament network that extends throughout the cytoplasm from the nucleus to the plasma membrane. They are also found in the nucleus where they form the nuclear lamina. While the large multigene intermediate filament protein family has more than 45 members, that are expressed in a cell and tissue specific manner (411), analysis of the amino acid sequence of the central rod domain has placed them into 6 classes (341).

Type I and Type II

Acidic and basic keratins form obligate heterodimers containing one type I chain and one type II chain. They are the most variable of the intermediate filaments and more than 15 isoforms of each type have been identified. Keratins are found in epithelial cells (419).

Type III

Vimentin is found in cells of mesenchymal origin and many cells in tissue culture, while desmin is a muscle specific intermediate filament that is switched on when myoblasts differentiate to myotubes. Glial fibrillary acidic protein (GFAP) (50kDa) is present specifically in astrocytes and non myelin forming peripheral glial cells while peripherin is expressed in peripheral neurons (419).

Type IV

In vertebrate neurons there are 3 neurofilament isoforms of varying Mr, NF-L (68 kDa), NF-M (160 kDa) and NF-H (200 kDa) (260). In addition, α internexin, is an early embryonic, brain specific intermediate filament (33,331,419).

Type V

Nuclear lamins A, B and C form a highly organized two dimensional filament sheet beneath the nuclear membrane. They contain a nuclear localisation site, and are subject to post translational modifications, which increase their hydrophobicity and target them to the inner nuclear membrane (296,419).

Type VI

Nestin is co-expressed and co-assembled with vimentin in neuronal cells and is expressed in early development in undifferentiated multipotential stem cells (341,419)

In contrast to the complexity of their expression, intermediate filaments show similarities in structure with a common highly conserved, central α helical rod domain, 310 amino acids long (369). This features heptad repeats of amino acids (abcdefg)_n where a and d are generally apolar residues, and has a periodic distribution of charged residues, with alternate acid and basic zones, that will tend to assume a two chain coiled coil α helix. The heptad repeat is interrupted by short non helical linker sequences, that divide the rod into four distinct domains. The rod domain is flanked by non-helical N and C terminal regions, that

are variable in size and sequence, although they show considerable homology within a filament class.(250, 369, 411)

As the heptad repeat results in a strip of apolar residues along one side of the α helix, intermediate filament monomers spontaneously assemble into a coiled coil parallel chain dimer, which is stabilised by interaction of the apolar residues. Two anti parallel dimers then aggregate via their rod domains to form a partially staggered bipolar tetrameric protofilament which assemble successively in a staggered array to form intermediate filaments. The C and N terminals of the rod are critical for intermediate assembly. Intermediate filaments assemble spontaneously *in vitro*, without a requirement for other proteins or an energy source (419 , 418, 402).

Although *in vitro* intermediate filaments are only soluble in extreme conditions of pH and ionic strength, they show plasticity during cellular activities such as cell division, neurite outgrowth and myogenesis. Their assembly is dynamic and is likely to be modulated by phosphorylation with different kinases inhibiting intermediate filament assembly and facilitating disassembly. The phosphorylation site is at the basic N terminus, and the introduction of negative charges by phosphorylation negates the contribution of basic arginine residues in the N terminal head domain to intermediate filament formation. For example, the keratin network is reorganized upon hepatocyte cell stimulation with EGF and this correlates with its phosphorylation, while *in vivo*, phosphorylation of vimentin and desmin parallels their disassembly at mitosis. Similarly, at mitosis transient phosphorylation by p34^{cdc2} kinase causes the nuclear lamina to reversibly disassemble into tetramers, with lamins A and C becoming soluble and dispersing through the cytoplasm, while lamin B remains associated with the vesicular remnants of the nuclear membrane. Dephosphorylation at the end of mitosis induces repolymerisation of the lamina proteins as a new nuclear membrane reforms (317). There also appears to be a considerable exchange of subunits from a small pool of unpolymerised subunits along the length of existing intermediate filament networks, with a dynamic equilibrium between a soluble pool and polymerised filaments. Furthermore, nascent chains of vimentin associate with intermediate filaments in cell free preparations indicating that there is a pathway for assembly of vimentin into polymerised intermediate filaments during their translation, in addition to the

continuous incorporation of newly synthesised proteins into existing networks which appears to be required to maintain the integrity of these networks (403, 103, 369).

The presence of intermediate filaments in most cell types and their tissue and developmental stage specific expression suggest that they do have vital roles *in vivo* but this has been difficult to define (335). Keratins have an important structural role in maintaining the integrity of the epidermis, and neurofilaments in axons running alongside microtubules provide support and structural rigidity (33). Intermediate filaments are also thought to form structural links between the plasma membrane and the nucleus, via, for example vimentins association with ankyrin/plectin at the plasma membrane and nuclear pores via lamin B.(369) This may help to maintain the position of the nucleus in the cell. Interactions between lamins A and C and chromosomes may have a central role in the organization of chromatin, and lamins provide structural support for the nuclear envelope, and have been implicated in the regulation of differential gene regulation, nuclear structure and reformation of the nucleus after mitosis (411, 296).

Changes in the primary structure of intermediate filaments are associated with several skin diseases. A single point mutation in the gene sequence encoding the rod domain of a keratin present in the basal cells of human epidermis results in defective keratins *in vitro* and large lumps of non functional keratins *in vivo* lead to the blistering skin disease epidermolysis bullosa simplex (227).

A diverse array of intermediate filament associated proteins (IFAPs) modify intermediate filament function and are responsible for the diversity of intermediate filament organization and function. These molecules are often cell and tissue specific.

For example, plectin can interact with and cross link a number of intermediate filament proteins (such as vimentin, desmin, neurofilaments, GFAP). Plectin also binds to MAPs, and may therefore connect intermediate filaments with microtubules. In addition, the high affinity of plectin for fodrin suggests that it has a role in linking intermediate filaments to the plasma membrane. Paranemin and synemin are intermediate filament cross linking proteins identified in muscle and associated with vimentin and desmin with filaggrins (a family of epithelial specific proteins) implicated in aggregating keratin filaments into bundles. Desmoplakins I and II and desmoccalmin bind to intermediate filaments and

anchor them at desmosomes, the site of intermediate filament : plasma membrane interaction (369,116). The function of epinemin, which associates with vimentin, is still unknown (246, 245).

4. MICROFILAMENTS

The third major cytoskeletal network is formed by microfilaments. These are 7 nm in diameter and are composed of linear polymers of actin molecules and actin associated proteins.

4 A. ACTIN

Actin was first discovered in 1942 by Straub in muscle tissue, where with myosin it forms the contractile apparatus responsible for force generation in muscle (230). It has a widespread distribution in muscle and nonmuscle cells and has been found in all eukaryotic cells studied so far. Actin has been highly conserved during evolution and is a very abundant protein (351). Actin monomers polymerise into microfilaments 7 nm in diameter and its assembly and organization into a wide variety of structures within nonmuscle cells are regulated by the coordinated action of 100 or more actin associated proteins (297). The actin system participates in many important cellular processes such as cell locomotion, phagocytosis, cytokinesis and cell surface receptor movements and intracellular transport. In addition enzymes and the protein synthetic machinery use the actin network as an organizational scaffold (414, 351, 236).

4 A i. Actin Isoforms

Comparison of primary structures shows that actin has been highly conserved during evolution (414); actin from species as divergent as slime moulds, yeast and mammals, have only small differences in their amino acid sequences, with *Acanthamoeba* actin differing from mammalian actin by only 15 out of 375 residues (501). This striking evolutionary conservation suggests that most of the residues are involved in the large number of specific interactions that are essential for actin to fulfill its many cellular roles. For example, each actin subunit within a filament is in contact with four adjoining monomers and also binds divalent cations and a nucleotide, so that each molecule is committed to six binding

interactions even before binding sites for actin associated proteins are considered (351, 426).

Most organisms express more than one actin isoform, with up to eight found in mammals and birds. The isoforms are encoded by a family of actin genes and are expressed in varying proportions in different cell types in a developmentally and spatially regulated pattern, depending on cell type (371,180). Each isoform has a distinctive distribution and an individual cell can contain more than one isoform. This tissue specific expression is conserved across species, suggesting a functional basis for isoform multiplicity (371,180). Actin isoforms can be subdivided into two classes, muscle and nonmuscle and are designated α , β and γ according to their isoelectric point, with α being most acidic (134). The muscle actins include α skeletal, α cardiac, α and γ smooth muscle actin. The predominant muscle actin in vascular smooth muscle is α smooth, in enteric smooth muscle, γ smooth. In smooth muscle, unlike skeletal muscle and cardiac muscle, 50% of the actin is comprised of nonmuscle isoforms, cytoplasmic β and cytoplasmic γ , which, with smooth muscle α , are the major actins in nonmuscle cells (371). Two additional isoforms that co-migrate with cytoplasmic actins have recently been found in intestinal epithelial cells (371). Muscle actins are similar to each other but distinct from cytoplasmic actins. Muscle and cytoplasmic actins differ by 23 out of 375 amino acids which is greater than the difference between mammalian cytoplasmic and *Acanthamoeba* actin (454). The small differences in amino acid sequence of actins found in isoforms are conserved across species (351,414, 371).

Differences in isoactin polymerization kinetics and binding affinity for actin binding proteins have been observed and are likely to depend on sequence differences. Nonmuscle actin binds with higher affinity to profilin and thymosin β 4 than muscle actins and this may lead to structures selectively enriched in one isoform. Cofilin, ADF and thymosin β 4 bind to β and γ nonmuscle actins preferentially and this may promote the assembly of newly synthesized α actin into myofibrils in maturing skeletal muscle. Studies with different actin isoforms have shown that they are capable of being used in isoform specific manner in cells, the ability of actin isoforms to activate specific myosin isozymes can vary depending on which actin is used with which myosin. The most effective activation is obtained when

actin and myosin are obtained from the same tissue. If different isoforms exist within a cell, exchange of actin and myosin could account for the different motility and the different cell shape (371).

The acidic N terminus in the most variable region of actin (the difference here accounts for the distinctive pI of isoforms) although there are several differences in primary structure scattered throughout the molecule (180). The N terminal difference peptide lies at the surface of the actin molecule in both monomer and filamentous forms and has been found to interact specifically with a number of actin binding proteins including myosin (180). It is likely therefore to modulate isoform specific functions, produce protein filaments with different stabilities and influence actins interactions with actin binding proteins. Its functional importance has been demonstrated by the removal of terminal acidic residues, which yields an actin which spontaneously assembles into filament bundles. The addition of two negative residues to the N terminal of yeast ACT1, which does not normally activate muscle myosin ATPase activity, restored activity to half that of skeletal muscle actin (180). There is circumstantial evidence for the functional diversity of actin isoforms, such as the switch in expression pattern from nonmuscle to skeletal muscle isoforms as myoblasts differentiate (180, 371). A role for nonmuscle actins in muscle cells in cell proliferation and movement is suggested by the observation that while aortic smooth muscle cells produce α vascular when quiescent, on migration and proliferation following injury, nonmuscle β and γ actin synthesis increases (180).

The suggestion that muscle and nonmuscle actins are functionally different has been supported by localization studies with isoform specific antibodies. Differential localization within in a single cell has been demonstrated in skeletal muscle cells where nonmuscle actins are excluded from the contractile apparatus and are preferentially localized in a subsarcolemmal array of actin filaments (371), and in vascular smooth muscle and pericytes, which restrict muscle actin to stress fibres while smooth muscle actins are found in regions of actively advancing cytoplasm. Nonmuscle actins are found in the cortex and non-polymerized actin pools (371).

The relative expression of β and γ actins may also be of functional significance, as the ratio differs between different tissues and through the cell cycle (180).

Nonmuscle actins seem to have a role in regulating cell motility and cytoskeletal architecture. In endothelial cells moving in response to injury, β actin is exclusively distributed within actively motile membrane domains (lamellae, ruffles, advancing pseudopodia) suggesting that control of β actin nucleation and filament assembly from the plasma membrane influences forward protrusions. Rac which induces membrane ruffling may be a putative modulator of β actin membrane dynamics (180). β actin localization and enrichment at the peripheral regions of motile cells suggest that it has a function in the organization, remodelling and spreading of cell membranes. The altered expression of actin isoforms in transformed cells may in part be responsible for the altered morphology and behaviour of these cells (180).

In situ hybridization reveals a specific distribution of β actin mRNA within β actin enriched motile regions. Functional diversity of β and γ non muscle actins are also observed with isoactin gene replacement, where over expression of β actin in myoblasts causes an increase in cell surface area and loss of stress fibres. Overexpression of γ actin causes transfected cells to round up and to decrease in size. It is thought that differences between actin isoforms may be due in part to differences in the untranslated region of mRNA as well as differences in protein sequence, for example, their localization within the cell. Alterations to transfectant cell morphology can be influenced by the presence or absence of noncoding regions in the transfected γ actin gene. (264) A mechanism to generate differences in isoform function could be the specific positioning of newly translated actin within the cell and it has been shown that β and γ actin are differentially located in myoblasts (183).

α actin seems to be mainly involved in contraction and are preferentially incorporated into myoblasts and stress fibres, β actin with cell motility and γ cytoplasmic actin with cytoskeletal array (180).

A divergent actin, ACT 2 (a yeast homologue of the conventional yeast isoactin ACT 1) has been found in *S.cerevisiae* and *S. pombe*. (257, 180, 396). While ACT 1 is 90% identical to mammalian actin, ACT2 which is larger and more basic, has only 35-47 % identity with ACT 1 and mammalian actin and cannot polymerise. In these actin like proteins ATP and divalent cation binding sites are conserved although it appears that some of the sites

of actin :actin interaction are not. Vertebrate cells also contain an actin like protein, actin RPV in dynactin complex (258) and centractin (70) which associates with centrosomes.

4 A ii Post-translational Modifications

Post-translational modifications of actin include acetylation of the N terminal residue (N-acetyl, glutamate or asp) (for which no function has been demonstrated) and methylation of His 73 (retention of this implies an essential role for this amino acid) (348, 371, 351). Furthermore in some insect flight muscles a proportion of actin is ubiquitinated (348).

In *D.discoides* the concentration of profilin and its affinity for actin are too low to account for the G actin pool, and thymosin β 4 has not yet identified in this organism. The amount of polymerization competent actin may be regulated by direct modification of G actin, resulting in activation of the monomer or modification of its interaction with sequestering proteins. In *Dictyostelium*, 10 - 15% actin is phosphorylated on tyrosine and serine residues when starved cells are introduced into nutrient media. The increase in tyrosine phosphorylated actin correlates with a decrease in F actin , disruption of cell morphology and cell motility and reduced substrate adhesion, suggesting that tyrosine phosphorylation alters the polymerization ability of actin and/or its interaction with other proteins (75,191) Actin phosphorylation has not been reported in vertebrates. Actin from *A proteus* can be phosphorylated in vitro by an endogenous crude kinase. Phosphorylation is specific for amoeba G actin or a profilactin complex. F actin is not phosphorylated and G actin phosphorylated in the presence, but not the absence, of profilin is polymerization incompetent. It is possible that phosphorylation increases the affinity of profilin for actin and induces the formation of a stable complex. Phosphorylation of the pointed end domain of actin bound to fragmin in *Physarum* by an endogenous kinase activity inhibits the nucleation and capping activities of the complex (75, 129).

4 A iii G Actin Structure

As normally isolated, monomeric (known as globular or G) actin consists of a single polypeptide chain of 375 amino acid residues, with Mr 43 kDa (219). A slight excess of acidic residues results in an isoelectric point of 5.5. Each monomer binds a nucleotide

(ATP or ADP) and divalent cations, generally believed to be Mg^{2+} in the cell, at specific sites (348, 219). Removal of the nucleotide and divalent cations renders G actin unstable and prone to proteolytic degradation. Each monomer has a single high affinity site and multiple low affinity binding sites for divalent cations. The complete amino acid sequence for actin has been determined, and comparisons of primary structures show that the actin molecule has been highly conserved during evolution.(41)

X-ray diffraction studies of a 1:1 co-crystal of G actin with DNase I have revealed the three dimensional structure of the actin-DNase I complex to atomic resolution (2.8 Å) (219).

The actin subunit (overall dimensions 3.7 x 4.4 x 6.7 nm) is a globular, bilobed molecule consisting of a large and a small domain (although they are now known to be similar in size)(219) separated by a deep, pronounced cleft, in which the nucleotide and its associated divalent cation are located (351, 219). The connection between the two domains is narrow and thought to be flexible. Each domain is further subdivided into two sub-domains, so that the small domain is composed of subdomains 1 and 2, and the large domain of subdomains 3 and 4 (219). These 4 subdomains are held together mainly by salt bridges and H bonds to the phosphate groups of the bound nucleotide and to its associated cation, at the centre of the molecule (41).

Both N and C termini are in the small domain and the whole central section resides in the large domain (219). The termini are near each other, and lie at the surface of the actin molecule facilitating their interaction with other molecules. The N terminal difference peptide is located on the surface of the actin molecule. Both domains have a central hydrophobic region of 5 stranded β pleated sheets, which confer nucleotide and cation binding abilities on actin, surrounded by 2 or 3 α helices (219). Because subdomain 2 has significantly less mass than the other three domains the actin molecule appears polar in the direction of the cleft (with the pointed end at opening of cleft, and the barbed end at base) (41). Many of the properties of actin, such as the binding of numerous proteins to its N terminal segment, inhibition of polymerisation by DNase I binding, exchange of the bound nucleotide, and the sensitivity of the cys 374 probe to its polymerisation can be explained by considering the atomic structure of actin (364).

The atomic model assumes that the conformation of G actin is not significantly altered by complexing with DNase I and that this binding is only likely to perturb the DNase I binding loop and the nucleotide cleft that it bridges. This has been tested by examining the structure of G actin in complex with other proteins which bind to different sites, and there is good qualitative agreement between this actin model and ones derived from electron microscopy studies (364).

4 A iv Actin Filament Structure

Under the correct conditions (10mM monovalent salts or a lower concentration of divalent cations) monomeric actin can reversibly polymerize into filaments (F actin) 7nm in diameter and of up to several μm in length, and this ability of actin to polymerise into filaments is fundamental to its biological activity (414). Monomers are uniformly oriented within the filaments which are therefore polar with structurally different ends. This polarity is essential to the function of actin filaments, and is used by the cell to promote the spatial organization of F actin and to control the movement of motor proteins. The addition of myosin subfragment 1 (S1) to F actin allows this polarity to be visualized as an arrowhead-like S1 decoration with barbed and pointed ends of actin filaments easily distinguishable (41, 414, 61, 301).

A model of the F actin filament has been constructed from the atomic structure of the actin monomers to fit with the observed X-ray fibre diagram from oriented gels of actin complexed with phalloidin (300,187). The actin filament can be described as a one start, left handed "genetic" helix with thirteen subunits per six left hand turns of 5.9nm pitch, but by regarding the actual subunit contact pattern, the filament is clearly built as a two start, right handed "long pitch" double helix of 71.5 nm pitch, with thirteen subunits in two strands together between each crossover point and thirteen subunits in each strand per complete turn of the helix. The two long pitch helices are axially staggered relative to each other by 2.75nm, half the subunit repeat, and crossover each other every half turn of the long pitch helix (35.8nm). Because the actin molecule is rather flat, the filament is widest where the two strands cross each other, that is where subunits lie on top of each other, and narrowest where they lie side by side (41, 414, 61) The actin subunits are arranged with

their long axes aligned roughly along the genetic helix, almost perpendicular to the filament axis (300,187). Within the filament, each actin subunit interacts with four of its neighbours and contacts seem to involve all four subdomains(426). Hydrophobic and electrostatic forces contribute to the non covalent bonds that hold the subunits together (414). Contact between subunits within the polymer is made both along and between the two long pitch strands. Occasionally the two strands separate and unravel locally, suggesting that the strongest contacts are between subunits along the long pitch helix (41). These contacts occur between the large domains of adjacent subunits, and in addition the C terminus and DNase I binding loop establish further long pitch contacts between the small domain of one subunit and the large domain of an adjacent subunits. Contact between the strands is mediated primarily by the insertion of a hydrophobic loop which extends across the filament axis into a hydrophobic pocket formed by the interface of two adjacent subunits of the opposite strand (41,187).

In electron micrographs, filaments of actin appear to be straight, spanning several μm . However, these filaments are not rigid but can flex along their length and twist around their long axis (208). This can lead to random disorder in the helical arrangements of subunits (414). Variability in flexibility and torsion (modulated *in vivo* by actin associated proteins (61) may be important for the formation of certain actin structures. For example, the formation of bonds by associated proteins that hold bundles of filaments together (for example in stress fibres) may require filaments that can be locally twisted (413). Actin filaments will buckle under compressive forces, but are resistant to extensive forces as found in sarcomeres. To achieve stiffness and mechanical integrity, actin filaments are crosslinked into larger arrays, such as parallel bundles and orthogonal gels (301)

4 A v Actin Polymerization

Actin polymerization from monomers proceeds in four reversible steps and, *in vitro*, is dependent on solution conditions of temperature and ionic composition, and can be induced by the addition of 50-100mM monovalent salt (KCl) or 0.5-2mM divalent salts (414, 351)

(a) Monomer activation

Monomer activation involves the binding of Mg^{2+} to moderate affinity sites of an ATP-G actin monomer, which induces a conformational change in the monomer, to a form that is inactive in polymerization (351). This was first detected as a resistance to proteolysis of monomer bound to cation and nucleotide. Following activation by Mg^{2+} , actin forms nuclei ten times more rapidly than unactivated actin. Activated Mg^{2+} actin also elongates actin filaments more rapidly than Ca^{2+} actin. ADP actin binds Mg^{2+} , but does not change conformation, nor become activated as ATP actin does. Mg^{2+} ions bind specifically, replacing Ca^{2+} (351). High concentrations of monovalent salts will induce polymerization without binding to specific sites.

(b) Nucleation

This step describes the association of several actin monomers into oligomers which have a higher probability of growing into filaments than decomposing into monomers, and is energetically unfavourable. Under normal polymerizing conditions, a dimer is unstable because the rate of dissociation is almost as fast as the association rate. However, when a trimer is formed, further molecule addition becomes more favourable than dissociation. Formation of a trimer is therefore required for further elongation. There is little direct evidence for the existence of dimer or trimer intermediates as during spontaneous polymerization, the concentration of dimers and/or trimers is very low and their lifetime is quite short, and most of what is known has been learnt from kinetic modelling. The high rate of dissociation causes most to disintegrate quickly and elongation is so fast that trimers grow into longer, more stable filaments. The nucleation step is slow and rate limiting because the reactions are unfavourable and therefore the time course of spontaneous polymerization of monomers *in vitro* is sigmoidal, with a lag phase at the beginning. This initial lag phase can be eliminated by seeding the solution with preformed actin filaments. Monomers will preferentially add to pre-existing filaments rather than form new nuclei so that filament assembly of purified actin *in vitro* will favour filament length over number. The nucleation rate is dependent on the solution conditions, for instance, nuclei formed in the presence of Mg^{2+} are more stable than if Ca^{2+} is present, because of the conformational change induced by Mg^{2+} . ATP actin forms nuclei faster than ADP actin.

Therefore, tight binding of Mg^{2+} ATP or Ca^{2+} ATP determines actin conformation and the nucleation rate (414, 351, 230, 348).

(c) Elongation

Once a stable nucleus has formed, the addition of monomers occurs rapidly, until the concentration of the monomers falls to the critical concentration, at which the net addition of monomers equals the net loss. Below the critical concentration, there is net dissociation of monomers from filaments and the filaments shorten. Above it, the association rate is greater, and the filaments lengthen, until the critical concentration and apparent equilibrium are reached. Thus, elongation refers both to the association and the dissociation of actin monomers from both ends of the filament.

Because actin filaments are polar, (and the terminal subunits are likely to differ in conformation from free monomers), association and dissociation reactions differ at the two ends of the filaments, as the polar monomers bind to the two ends at different rates. The rate of subunit association is rapid at both ends, but is faster at the barbed end, which therefore elongates more quickly. The rate of dissociation is relatively slow at both ends. The rate and extent of polymerization also varies greatly, depending on whether the nucleotide bound to the monomer is ADP or ATP. ATP actin subunits associate more rapidly than ADP subunits at both ends and the addition of both ADP and ATP is faster at the barbed end than at the pointed end. Therefore, ATP actin addition at the barbed end is rapid and limited only by diffusion; and polymerization is more favourable with ATP actin. At the barbed end, the rate of ADP dissociation is quite rapid and greatly exceeds ATP dissociation. At the pointed end, ATP dissociation exceeds ADP dissociation. After ATP hydrolysis and P_i release, ADP generally remains bound to subunits in the actin filaments and ADP is exchanged for ATP only when the monomer returns to solution.

For ATP actin, the critical concentration of the barbed and pointed ends may be equal or different, depending on the solution conditions. In the presence of Mg^{2+} , there are substantial differences between the critical concentration of the two ends. Under physiological conditions, the critical concentration is $0.1\mu M$ at the barbed end and $0.6 - 1.5\mu M$ at the pointed end. As a result of this difference, at steady state, the free monomer

critical concentration falls between these two values and net addition at the barbed end equals net loss at the pointed end, and there is a net flux of actin monomers through the filament, a phenomenon known as treadmilling, which requires energy provided by ATP hydrolysis; evidence for which has been provided by adding radioactive labelled monomers to an equilibrium of filaments and subunits. The flux of monomers is damped by the slow rate of dissociation of ADP actin at the pointed end (414, 351, 230,348).

(d) ATP Hydrolysis

Polymerization of ATP-G actin is associated with the hydrolysis of ATP to ADP, which consists of two temporally distinct steps, namely:

(a) irreversible cleavage of ATP followed by

(b) reversible slow release of P_i , which remains bound to the polymer for some time after cleavage.

Excess P_i , added to a solution of actin will rebind ADP. Elongation does not require ATP hydrolysis, which lags behind monomer addition, as ADP actin will polymerize, albeit slowly, as will G actin bound to a non hydrolysable ATP analogue. However, the nucleotide composition of the terminal filament subunits has an important effect on the rate and extent of elongation. For ATP actin, as ATP-G actin will add more readily to a filament with terminal ATP subunits.

In filaments composed of Mg^{2+} ATP F actin, the cleavage of ATP occurs essentially vectorially during polymerization, resulting in clear boundaries between F-ADP, F-ADPP_i and FATP subunits, so that actin subunits in the interior of filaments have ADP, but the first few terminal subunits (at the barbed end) have ATP at fast growth rates, F-ADPP_i at moderate rates and F-ADP under depolymerizing conditions. Under optimal polymerization conditions, the addition of G-ATP actin exceeds the rate of hydrolysis of newly incorporated ATP monomers (which dissociate more slowly than F-ADPP_i) and forms a stabilizing ATP cap to which further ATP subunits are readily added. F-ADPP_i capped polymers (which at steady state are present at the barbed ends only because polymerization at the pointed end is too slow for them to accumulate) lose subunits five to ten times more slowly than FADP polymers. Thus the intermediate F-ADPP_i state is very stable. A large

free energy change is linked to the release of P_i , and is related to destabilization of actin : actin interactions, possibly via a conformational change.

As the rate of addition of new subunits decreases, the stabilizing ATP cap is lost more often and ADP terminal subunits are exposed. Rapid depolymerization follows, until rescued by the addition of new ATP subunits. Delayed ATP hydrolysis promotes monomer addition when actin is assembling and facilitates rapid depolymerization on exposure of ADP monomers and thus enables dynamic filament turnover which is costly in terms of ATP and therefore *in vivo*, when this is not required, the barbed ends are blocked with capping proteins, which stabilize the filaments (230, 348, 60, 61,301).

(e) Annealing of actin filaments

While monomer exchange at filament ends determines the growth and stability of filaments, it is possible that the length and number of actin filaments and hence viscosity of actin solutions can also be influenced by fragmentation and annealing. Although relatively resistant to breakage under small sheer stresses, actin filaments are flexible and can break (due to thermal or fluid motion of the sample) and this may happen, for instance, during muscle contraction or cell movement. Since actin viscosity is dependent on concentration, together with the average length of the filament and cross-links between them, a simple method to alter the consistency of an actin rich cytoplasm is to vary the number and length of the filaments. Breakage of filaments would expose ADP actin subunits, leading to some depolymerization, but also to an increased number of filament ends, which would affect by the critical concentration. Direct examination by electron microscopy confirms that following mechanical disruption, short actin filaments rapidly anneal end to end *in vitro* leading to increased filament length fragmentation. This does not appear to require ATP or exogenous monomers, but occurs through direct association of filament ends (311).

During the initial phase of actin self assembly, the contribution made to filament growth by annealing is likely to be small. However, at later stages, until hampered by the restricted movements of long filaments and therefore reduced diffusion, annealing may significantly alter filament growth by increasing the length and decreasing the number of filaments. Annealing may constitute an efficient mechanism for repairing breaks in filaments. Annealing after the action of specific actin severing proteins may contribute to the rapid

rearrangements of actin filaments associated with cell motility and morphogenesis (311, 348).

(f) Filament Flexibility

ATP F actin filaments unravel less frequently than ADP F actin filaments and are more compact, suggesting that ADP/ ADP_i filaments are less structurally and mechanically stable. It has been suggested that this may be due to ADP actin filaments adopting two conformational states so that ADP filaments formed by polymerization of ADP actin subunits are flexible, while ADP actin filaments made by hydrolysis of ATP actin filaments are rigid. The energy that is released on hydrolysis of ATP to ADP is trapped and stored within the filaments, and is available for mechanical work in the cell, for example by proteins that sever actin filaments (60, 207, 364). However, other studies have found no difference (353).

(g) Drugs Affecting Actin Polymerization

Drugs that affect actin polymerization such as cytochalasins, phallotoxins, cholera toxins and latrunculins, have been useful in the investigation of the functional role of actin in cells. Cytochalasins (ABCD and E) are a group of fungal toxins that have a complex mechanism of action. High affinity interactions with F actin filaments cap the barbed end, inhibit subunit association and dissociation and may even sever filaments. Low affinity binding to actin monomers leads to dimerization and hydrolysis of bound ATP. Cytochalasins also appear to have nucleating effect on G actin, so that binding to the barbed end of a small complex stabilizes it, and allows elongation from the pointed end. Cytochalasins may reduce the viscosity of actin filament networks by inhibiting the self association of filaments. Cytochalasins are able to penetrate living cells and produce complex, usually reversible, disruptive effects on actin filament organization and inhibit cell movement and cytokinesis (348, 230, 6) .

Phallotoxins are also fungal toxins which bind specifically to filamentous actin with a 1 : 1 phalloidin : actin monomer stoichiometry. They promote polymerization (reducing critical concentration) and stabilize filaments against depolymerization by inhibiting disassembly at barbed and pointed ends. Phallotoxins do not cross the cell membrane and must be microinjected into living cells where they inhibit cell movement and actin filament

reorganization. Fluorescent labelled derivatives are widely used for the localization of actin filaments in cells and tissues and for quantitation of polymerized actin (348, 230, 6).

Cholera toxins catalyze ADP ribosylation of actin and this ADP ribosylated actin loses its ability to polymerize and thus acts as a barbed end capping protein, altering filament polymerization and disrupting actin organization. Cholera toxins are able to penetrate live cells and provide a novel way to investigate the participation of actin filaments in cellular processes (348).

Latrunculins, which are isolated from sponges, promote actin depolymerization by forming a tight 1:1 complex with actin monomers that is unable to polymerize into actin filaments, rather than capping free filament ends. They are taken up rapidly by intact cells and disrupt the normal distribution of actin filaments (348).

4 A vi Mechanical Properties of Actin Filaments

The mechanical properties of a polymer solution depends on the concentration, length and stiffness of the polymer strands and on the presence and nature of cross links between them (208).

Solutions of filaments formed from highly purified actin are complex materials with both viscous and elastic properties (348), so that they resist flow and store mechanical energy. The entanglement of long filaments, or weak connections between the filaments in solution, gives them the properties of viscoelastic solids. Actin networks are thixotropic, that is they form gels if left to stand, are fairly stiff if deformed gently but become very fluid when stirred, due to breakage of interfilament connections. If undisturbed actin solution can recover their stiffness after perturbation (208, 351).

4 A vii Actin Filaments *In Vivo*

The properties of purified actin are greatly modified by the presence of a large number of actin associated proteins that are found in cells. *In vitro* the kinetics of polymerization favours the formation of a small number of very long actin filaments, that become entangled to form sterically immobilized networks with anisotropic bundles (417). *In vivo*,

however, many different proteins regulate the time, place and extent of actin assembly and the formation of a wide variety of actin structures (347,82, 351, 414)

4 A viii G:F Actin Ratios *In Vivo*

Actin is a major component in eukaryotic cells and although its concentration varies in different cell types, it is always one of the most, if not the most, abundant protein. In highly motile cells, such as neutrophils, lymphocytes and platelets, it constitutes 10-15% of the total protein at a concentration of 100-250mM (409). The actin content of fibroblasts accounts for 10% of the total protein (34). Such concentrations of purified actin would polymerize rapidly, leaving less than 0.1% (0.2mM) in monomeric form at salt conditions found *in vivo*, but a high proportion (60-70% in unstimulated neutrophils) of actin in non-muscle cells is unpolymerized (475). In cultured vertebrate cells, some of this G actin is localized into discrete foci (109). Although nucleation sites and filament ends are blocked by actin associated proteins, this alone cannot account for the high concentration of monomers and this pool of G actin must therefore be sequestered or buffered by proteins which bind reversibly to monomers and prevent their polymerization (430) Profilin was the first monomer sequestering protein to be discovered and although profilin : actin complexes dissociate on neutrophil or platelet stimulation when the F actin content increases twofold, its relatively low affinity for actin and its low concentration (insufficient to bind all of the high concentrations of G actin found in resting cells) relative to actin especially when its binding to membrane lipids (PIP₂) is considered, make it a poor candidate as the buffer for the actin pool in the cytoplasm in a typical motile cell (475, 409). Profilin, rather than inhibiting polymerization, may contribute to elongation by catalyzing the rate of nucleotide exchange of monomers and, in some circumstances, decreasing the critical concentration at the barbed end (430, 301)

Thymosin β 4 (FX), a 5kDa protein, which forms a 1 : 1 complex with actin monomers which are then unable to polymerize, is now thought to account for much of the G actin pool in resting cells, with a small contribution from profilin and other sequestering proteins such as cofilin and ADF (417). Microinjection of thymosin β 4 into normal rat kidney cells results in a dose dependent decrease in F actin as demonstrated by phalloidin staining. The

affinity of thymosin β 4 for actin, and its concentration relative to actin (it is present at levels similar to those of G actin in unstimulated PMN cells) are consistent to with its role as the dominant actin sequestering protein (301,374, 409, 475).

II

ACTIN ORGANIZATION AND FUNCTION

1. SKELETAL MUSCLE

Muscle under voluntary control which is capable of contracting rapidly is termed "skeletal" or "striated" muscle. It is composed of long thin fibres, each of which is a single large multinucleate cell (1-40mm long and 10-50 μ m wide) which arise during development by the fusion of mononucleate myoblasts. The bulk of the cytoplasm in these cells is made up of myofibrils, the structures responsible for muscle contraction and the generation of force. In the phase contrast microscope, a series of regular bands or striations are seen along the length of the myofibrils, dark (A) bands alternate with light (I) bands, and the I bands are bisected by darker Z discs. This banded appearance arises from the paracrystalline arrangement of thick myosin and thin actin filaments which make up the contractile apparatus and which is required for the production of force on a large scale (39, 5).

A section of the myofibril from one Z disc to the next is a sarcomere (the individual unit of contraction) which is composed of precisely arranged parallel thick (15nm) and thin (7nm) filaments. The bipolar thick filaments at the centre of the sarcomere (A band) are aggregates of about 300 molecules of myosin II packed in a regularly staggered array via their rod-like tails, with their globular heads protruding from the surface of the filament in a helical pattern. At the central M line, the thick filament is devoid of myosin heads and two sets of oppositely oriented myosin molecules join end : end via their tail regions. Therefore the myosin heads point in opposite directions at either end of the thick filament. (6) Thick filaments interdigitate at their ends with thin filaments (I band) which are composed of actin and actin associated proteins. The actin filaments are attached via their barbed ends to Z discs, an extended, densely cross-linked protein layer. Therefore, thin filaments on either side of the Z disc are of opposite polarity and the pointed end filaments overlap with thick

filaments. Thick filaments are within 13nm of adjacent thin filaments and the myosin heads of the thick filaments extend and cross-link to the adjacent thin filaments. As the sarcomere shortens in muscle contraction, the I band, but not the A band, is seen to decrease in length, the length of the individual filaments, however, does not change. Contraction is brought about as thin actin filaments slide relative to thick filaments (5,6,39).

The contractile activity of the myofibril depends on the actin and myosin filaments being held in correct alignment with each other. While myosin has some capacity to organize actin filaments into contractile bundles, the high degree of order and stability of the sarcomere is maintained by a number of accessory proteins, which are largely periodic in their distribution as determined by immunocytochemistry, reflecting the order of the sarcomere.

Actin filaments are anchored to the Z disc by α actinin which binds to the barbed ends of actin filaments and bundles the filament ends together in a characteristic square lattice. The muscle isoform is Ca^{2+} insensitive. Cap Z also binds specifically to the barbed ends of actin filaments with high affinity at the Z disc (474). At the muscle plasma membrane, the Z disc is anchored by proteins similar to those of the adhesion plaque, spectrin, ankyrin, vinculin and nonmuscle actin are assembled in specific sites called costameres. (39) Dystrophin (an absence of which causes Duchenne's muscular dystrophy) which binds to actin filaments is also found in this region closely associated with the plasma membrane (104).

Myomesin cross-links adjacent myosin filaments at the M line into a hexagonal array, while C protein, H protein and X protein are found at regular positions along thick filaments to stabilize this packing (39).

Minor proteins also contribute to the efficiency of the myofibril; a network of insoluble protein filaments provide continuity and elasticity to the structure, holding myofibrils together and attaching adjacent myofibrils to each other and to the plasmamembrane. Titin is a very large (1×10^6 kDa), long and highly elastic protein that lies parallel to thick and thin filaments and connects thick filaments to Z discs. Nebulin, another large protein (600kDa) is less elastic and is also anchored at Z discs and associates more closely with actin (and might act as a template for filament length). (436)

An intermediate filament protein, desmin, is found in most vertebrate muscle cells following their fusion from myoblasts to myotubes. It is present with actin at the periphery of Z discs, and by linking Z discs in adjacent myofibrils into a three dimensional network, desmin has an important role in maintaining the integrity of muscle and in synchronizing contractions of many fibrils in a muscle fibre. A network of desmin filaments anchored in costameres connects myofibrils to the plasma membrane (39).

Two actin associated proteins, tropomyosin and troponin modulate the interaction of myosin and actin via their interactions with thin filaments and permit skeletal muscle contraction to be regulated by Ca^{2+} . Skeletal muscle tropomyosin, a rigid elongate heterodimer lies alongside the long pitch helix on either side of an actin filament, inhibiting the interaction of myosin with actin, by steric blocking or altering the conformation of the actin monomers. The position of tropomyosin is controlled by troponin, a complex of three polypeptides, designated troponin T, troponin I and troponin C. Troponin T attaches the complex to tropomyosin, troponin I binds to actin and may hold tropomyosin in a position on the actin which, in active contracting muscle, is occupied by myosin heads, and therefore inhibiting actin : myosin interaction. On binding up to four molecules of Ca^{2+} to troponin C, conformational changes occur in the whole complex, which prevent the interaction of troponin I with actin. Tropomyosin is thus freed to shift its position on the actin filament. According to the steric block hypothesis, these movements expose sites to which myosin SI can bind. An alternative view is that rather than blocking myosin : actin binding tropomyosin prevents the progress of myosin through the cross bridge cycle. (39, 160, 503)

Myoblasts have a non-muscle cytoskeleton which, as muscle specific isoforms of actin, myosin and associated proteins, are expressed during development change into the highly organized striated myofibril of mature muscle cells. They have cortical actin filaments and structure, resembling stress fibres, which appear to be used as templates for assembling myofibrils (173,298).

Force Generation in Skeletal Muscle

Actomyosin contraction is driven by the interaction between myosin heads, projecting from thick filaments and the sides of actin thin filaments. This cyclical formation and dissociation

of cross-bridges between the two filament types is accompanied by ATP hydrolysis. This, and subsequent dissociation of hydrolysis products produces a series of conformational changes in the myosin heads that drive them in one direction and moves the filaments relative to each other, with thin filaments pulled towards the centre of the A band. ATP is replenished by phosphocreatine

In the rigour (ATP free) state, myosin is strongly bound to actin (at an angle of 45°). The binding of ATP to myosin weakens this attachment and myosin rapidly detaches from actin. The 'free' myosin hydrolyzes the ATP rapidly to ADP and Pi (the myosin rotates to a position perpendicular to the actin), but the release of these products is slow (and is a rate limiting step). Actin catalyzes the reaction by increasing the rate of dissociation of ADP and Pi, when myosin and associated hydrolysis products rebind weakly to the adjacent actin monomer (at an angle of 90°). The release of products is accompanied by a large drop in free energy and a conformational change in the myosin head, which is bound weakly to actin, and this creates tension. The myosin head moves (power stroke) and pulls against the actin filament (N.O.) The myosin light chains are not essential for the enzymatic (ATPase) activity of the actin heavy chain, but it has been shown that they play an important part in the transduction of the energy of hydrolysis into rapid movement, by the amplification of small conformational changes in the neck region of heavy chain myosin to which the light chains are bound (267).

The two heads on the myosin molecule move independently. Once an individual head is detached, it may be carried along by the action of the (up to 500) other heads in the same thick filament, so that at any one point, some heads will be attached and some detached. This requires a degree of flexibility or elasticity in the neck region of the myosin molecule. It has been postulated that movement of the myosin head may be due to the chaotic movement of thermal motion and that ATP hydrolysis produces a bias to thermal motion in one direction, perhaps generated by changes in charge distribution, elastic properties or the binding affinity of the myosin head. (5, 39, 391, 76, 65)

2. SMOOTH MUSCLE

Smooth muscle is found, for example in the stomach, intestine, uterus and arterial walls, and is composed of long, spindle shaped, mononucleate cells 200 μ m long and 5 μ m wide, surrounded by extracellular matrix. Electrical coupling via gap junctions between these cells ensures synchronous contraction which in smooth muscle is slow and sustained compared with skeletal muscle. Many effectors such as autonomic nerve impulses and hormone such as epinephrin are responsible for the stimulation of smooth muscle in various tissues. Smooth muscle is very like nonmuscle cells and their contractile apparatus share many features including structural organization and actin associated proteins such as calponin and caldesmon. These molecules may have similar functions in both cell types, that is controlling actin : myosin based contraction and maintaining organization. (5,39)

There are two distinct actin containing domains in smooth muscle cells, the cytoskeletal and contractile zones. The contractile zone is comprised of α or γ smooth actin muscle, smooth muscle myosin isoforms and proteins involved in the regulation of contraction such as calmodulin, caldesmon, calponin, tropomyosin, and myosin light chain kinase (39, 465). While contraction is thought to occur by relative sliding of thin actin and thick myosin filaments, the thick and thin filaments of smooth muscle are not assembled into discrete myofibrils with repeating sarcomeres and lack the high degree of organization found in skeletal muscle. The contractile apparatus is roughly aligned to the long axis of the cell and is dispersed throughout the cytoplasm with the barbed end of the actin filaments attached to a cytoplasmic or plasma membrane associated dense body/plaque, while the pointed ends interdigitate with myosin thick filaments. The opposite end of the myosin filaments overlap with another set of actin filaments, which associate at their opposite end with another dense body. The contractile apparatus is anchored ultimately to the plasma membrane and coupled to the extracellular matrix through a membrane associated dense plaque. While cytoplasmic dense bodies are amorphous structures analagous to skeletal muscle sarcomeric Z line containing α actinin, the plasma membrane associated dense bodies which connects groups of cells together are analagous to an adhesive plaque of fibroblasts and contain α actinin and vinculin . Thick myosin filament formation can be regulated and thick filaments are labile in relaxed muscles. This may control the spatial and

temporal localization of myosin in cells, and it is possible therefore that the contractile apparatus can be rearranged to produce contractions in different directions as required, in a manner similar to that seen in nonmuscle cells, with the number of myosin filaments varying with physiological state and the degree of contraction. The aggregation and activation of myosin II is controlled by calcium activated myosin light chain kinase (MLCK) phosphorylation of the 20kDa myosin light chain which converts the myosin molecule from a folded, inactive configuration to an extended form capable of assembling into filaments, interacting with actin and with ATPase activity. (465, 398, 438)

Because of the lability of smooth muscle filaments and their potential for rearrangement, the packing of myosin filaments within a native filament is unclear although it has been suggested that smooth muscle myosin assembles into side polar ribbon shaped filaments, where each face consists of molecules arranged with the same cross bridge polarity with heads projecting from the end. Crossbridge polarity does not reverse at the centre of a bipolar filament, but continues along the filament length and crossbridges on each side can travel towards the barbed end of any actin filament with suitable polarity and effect the extensive contractions typical of smooth muscle (438, 6).

The cytoskeletal zone of smooth muscle contains non muscle actin isoforms, together with associated proteins such as tropomyosin, filamin, α actinin and vinculin and the intermediate filament proteins desmin and/or vimentin. These form an interconnecting network linking dense plaques, thus integrating the contractions of separate contractile elements, limiting the extension of the cell, distributing tension throughout the cell and hence contributing to the elastic recoil ability of smooth muscle (39, 465).

Smooth muscle generates comparatively greater force than skeletal muscle while consuming less energy. Following muscle stimulation, cytosolic Ca^{2+} levels increase and calmodulin binds calcium inducing a conformational change the molecule and exposing binding sites for MLCK, which has a high affinity for calcium calmodulin. This interaction activates MLCK, enabling it to phosphorylate residue ser19 on each of the two 20kDa regulatory myosin light chains. This relieves myosin light chain inhibition of actin-activated myosin ATPase, allowing myosin to interact with actin and triggers cycling of myosin cross-bridges along actin filaments, and the development of contractile force. The relaxation of

smooth muscle follows a reduction in calcium levels and the dissociation of calmodulin from MLCK, which reverts to an inactive form. Myosin, dephosphorylated by myosin light chain phosphatase, dissociates and remains detached from actin filaments.(465)

Changes in myosin light chain phosphorylation alone cannot account for all the changes seen in the contractile state of smooth muscle, such as tone, velocity of contraction and tension maintenance which are regulated, at least in part, by calcium sensitive, thin filament-linked regulatory factors, such as calponin, calmodulin and caldesmon.(465, 47)

Caldesmon is localized to thin filaments and binds actin, myosin, tropomyosin and calmodulin, and has an important role in this secondary, calcium sensitive regulation of actin : myosin interactions. It inhibits actin activated myosin ATPase activity by competing with the ADP P_i myosin complex for a binding site on the N terminus of actin and this inhibitory effect can be reversed by Ca²⁺ calmodulin binding to caldesmon. As a large (unphysiological) amount of Ca²⁺ calmodulin is required to release caldesmon from actin filaments *in vitro*, reversal of this caldesmon inhibition *in vivo* may be due to partial rather than total detachment from actin, a conformational change in caldesmon, or the presence of another as yet unidentified Ca²⁺ binding protein (465, 293).

This inhibitory effect of caldesmon is generally enhanced by tropomyosin since caldesmon has a greater affinity for actin : tropomyosin than pure actin. Tropomyosin may therefore increase the potency of inhibition by enhancing caldesmon binding or by propagating inhibition along the actin filament. (465, 293)

Smooth muscle is able to maintain tension during prolonged stimulation with low energy expenditure, even in low levels of calcium and with dephosphorylated myosin thick filaments. This is achieved by non- or slowly- cycling cross-bridges, the so-called latch-bridge state. The ability of caldesmon to form tension bearing cross-links between actin and myosin is thought to play a role in maintaining the latch-bridge state of contraction. (283) Phosphorylation of smooth muscle caldesmon is elevated in prolonged contractions and may therefore be involved in latch-bridge formation and maintenance. Furthermore, caldesmon will remain bound to actin, but interacts reversibly with myosin and may allow cross-bridge cycling.(465, 293)

In addition, caldesmon may have a structural role in smooth muscle, by tethering thick and thin filaments together, imposing the organization provided by Z and M line proteins in skeletal muscle, and holding filaments in register, so that they have the correct orientation and spatial distribution for the development of force. Caldesmon can dimerize *in vitro*, and this may allow it to cross-link actin filaments together. Filamin cross-linking may also increase the stability of actin filaments, playing a long term structural role that is unaffected by calcium.(465, 293)

Calponin is an additional calcium sensitive, thin filament associated regulatory protein in smooth muscle. It binds actin, tropomyosin and calmodulin, but not myosin. Calponin inhibits actin activated myosin ATPase without affecting myosin regulatory light chain phosphorylation.(465)

Despite its similarities to caldesmon, calponin is thought to regulate actin : myosin interactions via a different mechanism. Whereas caldesmon inhibits actin : myosin motility gradually (depending on calcium concentration), calponin regulation is 'all or nothing' and is unaffected by tropomyosin. Calponin does not bind to the N terminus of actin and therefore does not compete for binding sites on actin with ADPP_i myosin. As calponin binds to the C terminus of actin, it may induce a conformational change in actin and block initial weak ADPP_i myosin attachment, or inhibit subsequent strong binding of the ADPP_i myosin complex by competing for binding sites on the actin C terminus. Calponin and caldesmon regulation may therefore be complementary. The inhibition of ATPase activity by calponin is lost on phosphorylation which inhibits calponin : actin binding.

A model for calponin action has been proposed, whereby in resting muscle, with low calcium levels, calponin is dephosphorylated and bound to thin filaments. As calcium levels increase, calcium calmodulin kinase II phosphorylated calponin dissociates from thin filaments, MLCK phosphorylates myosin and tension develops. Since myosin light chain and calponin are phosphorylated by different kinases with different calcium sensitivities, as calcium levels fall, myosin light chains are fully phosphorylated, but calponin is mainly dephosphorylated and reassociates with the thin filaments, limiting the access of myosin to actin and inhibiting the ATPase cycle. The shortening velocity can therefore be fine tuned by altering the number, rate and cross bridge cycle by differential phosphorylation of

calponin and myosin. Calponin also cross links and bundles actin filaments and may make a contribution to the organization of thin filaments. (465)

3. NON MUSCLE CELLS

3 A CORTICAL ACTIN

The cortex of a cell is a thin (0.2 - 0.5 μ m wide) layer of actin filaments from which organelles are excluded, lying immediately beneath and attached to the cytoplasmic face of the plasma membrane (416,360). The concentration of actin in the cortex is very high - 50-100mg/ml and may be higher in the microspikes (393). If this formed a random network, it would be an immobile solid, but the cortex is highly dynamic. It is subject to the action of many actin associated proteins, which are found in the cortical region and control the structure/geometry of the actin network (393), enabling it to perform its many functions. By controlling the rate and extent of polymerization and therefore the filament length and number, and the degree of crosslinking, the viscosity of the cortex can be changed. This can be achieved by proteins which (1) promote nucleation (profilin, ponticulin, gelsolin) (2) inhibit nucleation (thymosin β 4), (3) inhibit elongation and annealing of filaments by capping barbed ends of filaments (gelsolin) (3) sever actin filaments (gelsolin, fragmin), (4) disrupt protein:protein interactions (calpain regulated proteolytic cleavage of talin following platelet activity). (415, 417, 62) The presence of myosin indicates that filaments can be organized the force generation necessary for motile activity (247).

The actin and actin associated proteins form a three dimensional network of short branching filaments, crosslinked at regular intervals (416). Linear actin filaments extending back into the cell and an additional interwoven array of filaments are also seen. This multilayered actin organization may reflect the fine control necessary for the cortex to carry out its functions. The cortical actin cytoskeleton may be continuous with stress fibres and other sub-cortical actin filaments (247). The actin isoforms in this region are predominantly β and γ , with β enriched in actively motile areas such as lamellipodia and membrane ruffles (371). The cortical cytoskeleton is anchored to the plasma membrane via a variety of proteins, for example, integral membrane proteins such as LSP-1 (214),

ponticulin (67), insertin (372), actolinkin and the EGF receptor (269,44). A number of proteins bind both F actin and transmembrane proteins and therefore tether the cortical cytoskeleton to the membrane. For example, dystrophin in striated muscle binds to laminin in the extracellular matrix via a glycoprotein complex (105), members of the radixin, ezrin, band 4.1 family which have membrane glycoprotein binding sites (44), α actinin can bind to integrin, and MARCKS to an unknown membrane site (269). ABP 280 links the actin cytoskeleton to GP Ib-Ix and increases membrane stability as demonstrated by the continuous blebbing of melanoma cells that lack ABP-280 (86). Other proteins including myosin I, α actinin and annexin I may link actin to membrane phospholipids (44). Proteins with SH₂ domains, such as tensin, can mediate cytoskeletal attachment to the membrane via their association with phosphotyrosine containing membrane receptors (44, 269). Further attachments are made via the spectrin actin network, which constitutes the membrane cytoskeleton of erythrocytes and is also found in almost all higher eukaryotic cells (269). While ponticulin and talin bind to the sides of actin filaments, others, such as actolinkin and insertin, bind to the barbed end of filaments, ensuring the polar orientation of filaments with respect to the membrane (269).

The cortical actin cytoskeleton, owing to its viscosity provides the cell with mechanical strength.(360, 416) It defines cell shape, and the rapid reorganization of cortical actin filaments, and therefore changes in its consistency in response to cell stimulation, leads to the active extension and retraction of surface structures, such as lamellipodia, filopodia and membrane ruffles and blebs that are essential for cell processes such as locomotion, chemotaxis and phagocytosis.(269, 44, 417) Shape changes that are seen on platelet activation, that is from discoidal to stellate, following fertilization of sea urchin eggs and morphogenetic changes in embryonic development are accompanied by reorganization of the cortex (62) and may involve rapid polymerization of G actin. As cells round up at mitosis they sprout surface structures that accommodate excess membrane. (43)

The cortical actin cytoskeleton may act as a regulative barrier to endocytosis and exocytosis, by obstructing the fusion of the plasma membrane with membrane bound organelles and vesicles, for instance in the exocytosis of synaptic vesicles at the neuromuscular junction of neurones. It may have a role in maintaining cell polarity, in

transmembrane signalling and cytokinesis (62, 44). It may interact with cell surface receptors and regulate their mobility and function. For example, acetylcholine receptors in skeletal muscle are restricted to the post synaptic membranes of neuromuscular junctions via their interaction with actin and associated proteins.(269, 43, 360)

In cells exposed to antibodies against surface components, the antibodies are seen to collect into a patch. Under the correct conditions (an energy source and temperatures greater than 4°C) patches will aggregate into a single large assembly (cap) at one pole of the cell, for example the posterior uropod of lymphocyte. Patches are carried into the cap via their association with the actin network, which moves in a concerted fashion. Capping involves the actin cytoskeleton as it can be inhibited by cytochalasins, and actin and actin associated proteins accumulate beneath the cap. (95, 342, 35)

3 A i Cell Surface Structures

Transient cell surface structures are seen in almost all higher eukaryotic cells and these microspikes, ruffles, and lamellipodia all contain actin based cytoskeletons. The functions of these structures are often poorly understood, because in many cells they are fragile and dynamic.(43, 415, 417, 476) In some cells, however, the cortical actin is less dynamic and is highly ordered, such as in the microvilli of the brush border found at the apical end of intestinal epithelial cells.

3 A i (a) Microvilli

The brush border of a single cell has about 1,000 microvilli, and the primary function of this surface structure is to increase the surface area of the gut lumen for digestion and absorption of nutrients. An individual microvillus has a diameter of 100nm and is 1-2 µm in length. The microvilli core contains 20-30 bundled F actin filaments of uniform polarity. Two thirds of the core bundle is surrounded by plasma membrane while the remaining one third protrudes into the cytoplasm as a rootlet and inserts into a second microfilament arrangement, the terminal web.(43, 266)

In the microvilli core bundle the actin filaments are bundled and cross linked by fimbrin and villin and their barbed ends, at the tip of the microvillus are embedded in a dense cap of protein that does not necessarily block elongation of the filament ends. (43, 266)

Villin is synthesized first and localized at the apex of the cell. It is probably involved in the assembly of the microvillistruature by nucleating actin assembly and capping the barbed end of the resulting structure as transfection of the villin gene into fibroblasts induces formation of microvilli and loss of stress fibres (122). Its bundling activity is also important for the formation and maintenance of this surface structure. It binds to actin in a calcium dependent manner; in the absence of calcium it bundles actin, but as calcium levels are increased it can sever actin filaments, and may be involved in the rapid destruction of actin bundles following cell death. Fimbrin is expressed concomitantly with the assembly of microvilli, and is thought to be the primary actin bundler. Cross linking by fimbrin results in formation of polar actin structures.(43, 266) Single headed brush border myosin I forms the lateral arms that attach the actin core bundles to the plasma membrane. It is not an integral membrane protein and while it is not clear how it is attached to the plasma membrane, two possibilities are that it binds to a 140 kD glycoprotein or to an acidic phospholipid. It may have a delivery function, transporting material to the apical membrane or a purely mechanical function as a spacer between the actin core and the membrane, allowing efficient diffusion. (43, 266, 486)

Ezrin is a minor component of the microvillus and while *in vitro* it binds to actin and phospholipids with moderate affinity in low salt, this activity is abolished in physiological conditions. There is a close correlation between ezrin phosphorylation and the generation of surface structures containing this protein. Its similarity to talin and protein 4:1 suggests it links the actin cytoskeleton to the plasma membrane (45). The terminal web of the microvillus contains the actin cross linker, fimbrin, myosin II, which generates cortical tension, maintaining microvilli in an upright stiff array on the cell surface, and tropomyosin, which competes with villin for the same actin binding site and therefore inhibits actin bundling by villin. Within the terminal web, core filament bundles are connected by TW260/240, a spectrin homologue which binds to calpactins, forming a sub membranous cortical layer. Intermediate filaments and micofilament bundles associated with the zona adherens form circumferential rings perpendicular to the microvillar actin bundles.(43, 266)

3 A i (b) Stereocilia

The stereocilia of auditory hair cells which act as receptors for auditory perception contain thousands of unipolar actin filaments highly crosslinked into bundles, in perfect axial register. They are longer than 30 μm , 1 μm wide and stiffer than brush border microvilli. They do not appear to contain villin or brush border myosin I, but are cross linked by fimbrin and this may explain why the filaments are more densely and regularly packed here than in the microvilli. The central filaments are longer and extend into randomly oriented dense meshwork of actin filaments cross linked by fodrin, a spectrin homologue. This cuticular plate anchors the stereocilia. Tropomyosin binds to rootlet actin filaments and filaments of the cuticular plate, stabilizing filaments, and preventing their depolymerization. It may inhibit actin : fimbrin binding allowing the rootlets to splay. (43, 227, 407)

3 A i (c) Growth Cones

Growth cones are highly motile structures found at the tips of elongating axons. They form during embryogenesis as neuroblasts differentiate and can be regenerated in adult axons following injury. While microtubules form the major cytoskeleton in the axon and in the rear and central region of the growth cone, actin and actin associated proteins (myosin, fodrin and synapsin I) are abundant in peripheral regions where they form a dense cross linked meshwork in the flattened palm of the growth cone, similar to the actin cytoskeleton found in lamellae. (106,157) Protrusive activity at the leading edge produces lamellipodia and microspikes. These contain unipolar actin filament bundles, cross linked by fimbrin, which extend into back into the growth cone (156). Regulatory proteins present in the growth cone include GAP 43, a calcium calmodulin binding, membrane associated phosphoprotein that has an important role in growth cone function, as it is only expressed in growth cones of developing and regenerating axons (117). Growth cones guide extending axons towards their distant target sites by precise pathfinding, which relies on their ability to recognise and react to extracellular cues from, for example, extracellular matrix molecules, by remodelling the actin cytoskeleton. If actin remodelling is blocked by cytochalasins, the axon extends blindly, as microtubules continue to promote axon growth (261). Growth cones are the precursors of presynaptic terminals, in which actin,

crosslinked by fodrin and synapsin, regulates vesicle access to their release sites at the presynaptic membrane. Post synaptic dendritic spines contain a filamentous actin core bundle. (117, 261, 36, 37)

3 A i (d) Transient Cell Surface Structures

Transient, more variable and less defined cortical structures extend and retract from the cell surface, especially from the leading edge of a motile cell, which is fan-shaped. A broad flattened region, the lamella or leading edge, extends in front of the thicker central perinuclear region in the direction of movement. (415). The actin cytoskeleton in these structures is similar to the cortex of a resting cell, a dense meshwork of actin filaments, associated with low Mr caldesmon, ezrin, spectrin α actinin filamin, talin and myosin I (415, 43). Thick filament bundles of actin, called arcs, of unknown function continually form at the leading edge, parallel to the cell perimeter and move backwards and their function is unknown (39). The actin filaments in the lamella may be oriented more or less in the direction of movement.(419) Remodelling of the cytoskeleton leads to the formation of microspikes, ruffles and lamellipodia which extend from the lamella, and new substrate contact sites are made within these structures (55, 417). Microspikes (or filopodia) are slender extensions, 0.1 - 0.2 μm in diameter and 5-15 μm in length that contain a single bundle of about twenty parallel actin filaments, crosslinked with fimbrin and oriented with their barbed ends towards the microspike tip. These bundles are continuous with actin structures deeper in the lamella.(416,415,269)

Lamellipodia extend from the anterior margin of the lamella in the form of thin veils of cytoplasm, less than 200 μm thick and about 5 μm long. They contain an orthogonal actin filaments, which may be more or less oriented in the direction of movement, interspersed with actin bundles or ribs (147), which have their barbed ends inserted into the leading edge. Low Mr tropomyosins are abundant in the dynamic lamellipodia (262), interacting with the predominant β actin isoform (371).

Blebs are spherical or hemispherical mounds, 2-10 μm in diameter, that expand from the leading edge of the cell and then flatten (415). They also appear on the dorsal surface of the cell during anaphase and in response to cytochalasin. They are also seen in transformed

cells in which expression of ABP is down regulated (29). They contain cortical microfilaments and may be the result of changes on cortical resistance as the meshwork structure is altered, for instance, during cytokinesis, or protrusion of pseudopodia. Their function is unknown, but they may accommodate excess membrane as cell morphology changes. (43, 416)

Lamellipodia and microspikes can interconvert (415), lamellipodia contain actin bundles and short microspikes often delineate their edge. Ruffles are formed by lamellipodia as they cyclically extend, lift up from the substrate, undulate and move backwards, before merging into the dorsal surface of the cell, 10-20 μ m from the leading edge. They therefore have the same composition as lamellipodia.(43) Increased ruffling is frequently observed on the surface of transformed cells (313). The function of ruffles is obscure, but it has been suggested that they may be involved in pinocytosis, cell locomotion or increasing cell surface area for particle transport (415, 269). Cells are most adherent at regions of active ruffling, at sites of lamellipodia and and microspike protrusion. These activities correlate with sites of substrate contact formation and ruffles may be specialized and actively concerned with the the accumulation and laying down of components necessary for making focal contacts. They are associated with nascent vinculin and talin containing contact sites, the precursors of adhesion plaques (368, 415)

Microspikes and lamellipodia appear to have a sensory function, similar to the filopodia of growth cones. (415) They extend and swing around, and if they adhere strongly, via the binding of specific cell surface receptors to substrate bound molecules, the cell edge flows out between the microspikes and they steer the cell towards adhesive regions. If they do not adhere, they fold back over the dorsal surface of the cell, and retract as they are carried towards the cell centre. Similarly, only some lamellipodia adhere to the substrata. (6) Cells are guided by extracellular matrix components, which are of varying adhesiveness and can therefore promote or inhibit migration. (39) Contact guidance is likely to be very important in development, where aligned extracellular matrix components can promote coordinated cell migrations, for example, neural crest cells follow pathways of extracellular matrix rich in collagen and fibronectin (39). Physical contours can also affect the migratory behaviour of a cell. They appear to be unable to cross a convex surface if the angle is too acute and

will align themselves and move parallel along the direction of least curvature, for example along a glass fibre or parallel with long axis of surface features, such as grooves and ridges (39).

Surface structures are involved in the phenomenon of contact inhibition of movement, so that when two migrating cells come into contact, the lamellipodia of both cells retract. The cells appear to join transiently, via a dense plaque, and protrusive activity such as lamellipodia formation and ruffling appears at another site on the cell margin. After a period the cells break their adhesion as they change the direction of migration. Contact inhibition has an important role in tissue development as the gross effect of such behaviour is to direct cells from regions of high cell density to regions of low cell density. It is also implicated in density dependent growth. In contrast to fibroblasts, epithelial cells do not migrate away from each other following contact, but form colonies. This is important in tissue formation and wound healing when sheets of epithelial cells move from the margins of a wounded area by extending lamellipodia. Once a continuous sheet of epithelial cells has covered the wound, all cell migration ceases and cell junctions are formed between cells.(2, 39)

3 A ii Erythrocyte Membrane Cytoskeleton

Red blood cells do not have a dense cortical actin cytoskeleton, but a highly specialised membrane cytoskeleton which forms a two dimensional network of spectrin, actin and associated proteins (269, 43). It is closely associated with and is attached to the cytoplasmic face of the plasma membrane at many points. This cytoskeleton provides the strength and structural support to the erythrocyte membrane that is essential for its survival in a high stress environment, while allowing the necessary flexibility for erythrocytes to travel along capillaries (269). The membrane cytoskeleton maintains the biconcave shape of the cell which is important for maximizing surface area. Erythrocytes deficient in spectrin or other components of the membrane are fragile and easily damaged or lysed (269, 43, 360).

Spectrin, an extended tetramer $(\alpha/\beta)_2$ is the structural basis of the membrane cytoskeleton. Rodshaped α and β subunits are aligned antiparallel in a heterodimer which joins head to

head to form a tetramer (200nm long) with F actin binding sites, in the tail region, widely separated. Short actin filaments of about 12 subunits link 5-6 spectrin tetramers together to generate a roughly hexagonal spectrin/actin lattice. Spectrin oligomerization and actin binding allow the formation of an extensive network. As spectrin binds to the side of an actin filament, each actin filament has many potential binding sites for spectrin. Protein 4.1 caps the pointed end of an actin filament and enhances the binding of spectrin to actin via its association with β spectrin. Tropomyosin and dematin, an actin bundling protein, also help to stabilize short actin filaments. Adducin, an F actin binding protein, stimulates the binding of further spectrin tetramers to F actin and has an important role in the assembly of actin. It also stabilizes the actin/spectrin attachments (269, 43, 360).

The flexible spectrin actin network is attached to the membrane indirectly via ankyrin, which binds the β subunit spectrin and the N terminal cytoplasmic domain of band 3, a transmembrane protein, an interaction which is stabilized by band 4.2, and directly via protein 4.1 which binds to glycophorin C, glycophorin A and with lower affinity to band 3 (269). Spectrin may also interact directly with integral membrane proteins. Spectrin does not form a rigid strut between the F actin filaments at junctional sites, as a flexible rod region and a hinge region in the molecule allow the molecule to extend and retract (360). Phosphorylation of spectrin (or associated proteins) can alter the rigidity and organization of membrane cytoskeleton by influencing its ability to change shape or associate with other proteins.

While the membrane cytoskeleton of erythrocytes has been studied most extensively, proteins analogous to spectrin such as fodrin, TW260/240 and to many accessory proteins have been isolated from almost all eukaryotic cells and structures similar to the erythrocyte cytoskeletal network are present in many other cell types, indicating that a spectrin-like cytoskeleton is of general importance. The spectrin actin network in non-erythrocyte cells may provide mechanical support to the plasma membrane and increase membrane stability, provide specific attachment sites for other cytoskeletal components and anchorage for cell surface receptors and ion channels. Non erythrocyte spectrin is localized in the cytoplasm as well as the plasma membrane, and can shuttle between the two sites, as, for example in lymphocytes following stimulation.(43, 269, 308, 360, 20)

3 B SUB CORTICAL ACTIN

3 B i Stress Fibres

In normal mesenchymal cells grown in tissue culture, subcortical cytoplasmic regions are often characterised by linear bundles of actin microfilaments called stress fibres (55). Stress fibres were first observed by phase contrast microscopy as dense structures spanning the cell. Immunofluorescence with anti-actin antibodies or fluorescent phalloidin has also demonstrated that there are finer linear microfilaments, which are also defined as stress fibres (56). By immunofluorescence and electron microscopy, non linear, randomly orientated microfilament bundles, (50 - 400nm diameter) and finer, single actin filaments can be detected and may be contiguous with stress fibres. Their role may be to connect dorsal and ventral stress fibres and form the meshwork of individual cross-linked actin filaments characteristic of many cells. Stress fibres normally exist in two distinct groups, the members of each group arranged in near parallel arrays and the two groups set at angles to each other. The majority of stress fibres in a cell lie parallel and adjacent to the cytoplasmic face of the plasma membrane in the ventral region.(56) They terminate and are anchored to the cell membrane at sites of the cell : substrate contact, the adhesion plaques (55) suggesting a role for stress fibres in cell adhesion (56). Another population of stress fibres, which are often thinner, arches over the nucleus on the dorsal surface of the cell.(56).

Labelling with SI myosin has shown that the actin filaments within a stress fibre are of opposite polarity (56) while immunofluorescence with antibodies to specific actin isoforms, and gene replacement studies, have demonstrated that stress fibres (like myofibrils) are predominantly composed of α actins (180,371). Stress fibres also contain bipolar aggregates of myosin dimers or short filaments suggesting that they are used for the generation of actin myosin based contraction (56,420). Furthermore, the periodic distribution of actin associated proteins in stress fibres, such as myosin, α actinin, tropomyosin and caldesmon, mimics the organization of the skeletal muscle sarcomere (56, 334). Myosin and tropomyosin are coincident but alternate with α actinin, while filamin (ABP280), in contrast, has a uniform distribution (56). In addition to force generating proteins, stress fibres contain proteins that are thought to regulate the contractile activity of

smooth muscle, such as myosin light chain kinase, calmodulin and caldesmon, whose distributions are coincident with myosin (56). Given the composition and organization of these components in stress fibres it was suggested that they are used for the generation of actomyosin based movement, and stress fibres were initially thought to have a role in cell motility and indeed stress fibres in permeabilised cells, separated from the surrounding cytoplasm by laser dissection, will contract in the presence of magnesium and ATP.(56, 53, 55) However, there is an inverse correlation between the presence of stress fibres and cell motility and rapidly migrating cells contain few, if any, stress fibres and, if present, they are restricted to the 'tail', which has tenacious adhesion sites (229, 259). Stress fibres are, rather, associated with non motile or slowly moving cells and an increase in substrate adhesion.(182,55,53,259). The rate of locomotion of well spaced cells is low (0.1 - 0.75 μm per minute), similar to the rate of change observed in stress fibre patterns (56). There does, however, appear to be a correlation between the presence of stress fibres and tension in a cell since only flattened stationary fibroblastic cells are able to deform (wrinkle) a thin latex substrate with stress fibres developing at right angles to these wrinkles (55). Highly motile cells such as leukocytes are, in contrast, unable to deform flexible substrates by their movements and it has been demonstrated that the protrusive activity at the leading edge of a moving cell does not require tensile force (55, 53). In motile cells, such as clusters of epidermal cells held in tension by micromanipulation needles protrusive activity is greatly reduced and the cell protrusions extend, if at all, only in the direction of the applied tension. This reduction in motility and protrusive activity is concomitant with the appearance of stress fibres within the cells (229). That tension held by contracting stress fibres can inhibit cell motility was further supported by an experiment in which an anti myosin antibody (known to inhibit myosin filament assembly in rats) was microinjected into chick fibroblasts. This caused the stress fibres to disassemble and ruffling motile activity to commence.(6) While tension exerted on the cell as it adheres to the substrate may alter a network of actin, pulling the filaments into parallel strands actin associated proteins such as filamin and α actinin, known to cross-link actin filaments may cause closer packing of filaments into a bundle (55, 229,53) The addition of other actin associated proteins such as tropomyosin and filamin would then stabilize these filaments and myosin, by cross-linking,

would also help maintain the stress fibre bundle integrity and complete the stress fibre structure (56). Such actin bundles would be capable of exerting force in the plane of tension or of moving slowly towards or away from it (229). Stress fibres exert an isometric, rather than an isotonic contraction during the development of tension following cell to cell or cell to substrate adherence (53,55). Since simple mechanical forces can regulate the motile behaviour of cells, such as tension transmitted by cell to cell attachments this tension may be important during embryonic development, influencing the arrangement of cytoskeletal elements in cells, which in turn, can modulate extracellular matrix components (56).

While stress fibres are common in cells *in vitro*, they also exist *in vivo* and have been found in cells from areas of high stress, for example, fish scleroblasts, endothelial cells of the arterial vascular system, splenic sinus, after wounding in corneal epithelium and in foetal tissue (55,56). Stress fibres are also prominent in endothelial cells, oriented parallel to the direction of the blood flow from regions exposed to high velocity flow, such as the left ventricle aortic valve and aorta. The role of this tension applied within cells by stress fibres would allow cells to maintain a flattened shape and to remain firmly attached to the substrate, and resist sheer forces (such as haemodynamic stress) that act on the cell in, for example blood vessels (56, 488). This has been substantiated by the finding that stress fibres can be induced in endothelial cells *in vitro* by exposure to fluid sheer stress equivalent to stress occurring in human arteries (120), while in scleroblasts, fibroblastic cells found in fish scales, stress fibres are found especially in cells near the edges of the scale or over radiating ridges, reflecting the greater need for cells to adhere to these regions of greater sheer stress forces (56). In foetal tissue stress fibres may have important implications for organism development such as tissue invaginations (2) and in modulating the orientation of the extracellular matrix and thus the pathfinding used by migrating cells in embryogenesis. These forces exerted by stress fibres may also be responsible for wrapping structures with collagen and aligning polarized matrix components leading to the formation of ligaments and tendons (56).

Stress fibres may play a role in compartmentalization of membrane proteins, thus creating cell surface domains in which receptors are segregated and have restricted diffusion. For

example, stress fibres co-distribute with cell surface fibronectin (196), whereas other membrane bound proteins, for example the Na^+/K^+ ATPase and macroglobulin are excluded from surface regions adjacent to stress fibres (56).

Stress fibres may also be involved in the maintenance of cell shape. Cells in which stress fibres are disrupted, either by cytochalasin or a transforming virus (461), show an altered morphology, changing from flattened, spread shape to a more rounded form (56).

Although prominent in well spread cells, stress fibres are dynamic structures(56), and are able to and must disassemble during, for example, cell division (181). Direct observation of live cells injected with fluorescent actin revealed rapid rearrangements of actin during the cell cycle and at steady state. Fluorescent actin is incorporated into the cortex, microspikes, pseudopods and stress fibres.

As cells spread, a polygonal network of actin filaments, geodomes may be seen, often above the nucleus. By immunofluorescence α actinin is present at the vertices of these geometric structures, while myosin and tropomyosin are excluded. These geodomes may be intermediates in stress fibre formation, since they are often found extending from vertices into the cell margins as cells respread following division. They may act as nucleation sites for stress fibres, or intermediates in the organization of contractile proteins but they are not an essential requirement for stress fibre formation (56, 249).

3 B ii Other Microfilament Bundles

3 B ii (a) Contractile Ring

The contractile ring is a transient bipolar array of actin filaments with their barbed ends attached to the plasma membrane, possibly via their association with radixin, at many sites around the equator of dividing cells. Interaction of cortical ring actin filaments (about 20 per bundle) with small bipolar myosin II filaments applies tension to the membrane, aligns the actin filaments, and (because the contractile ring is confined to a narrow equatorial band of cortex) constricts the cell locally, pinching the cytoplasm into two compartments, like a purse string. Actin filaments in the contractile ring are thought to recycle, intact, from other parts of the cell, moving first from the cytoplasm to the cortex and then to the cleavage

furrow. Accumulation of myosin II at the cortex and, especially near the equator where the furrow will form, is one of the earliest events in contractile ring assembly. (381)

Myosin, α actinin and filamin are found in the cleavage furrow. Spectrin, radixin, ezrin and moesin are also concentrated at this site and may attach the actin cytoskeleton to the plasma membrane. Microinjection of anti myosin antibodies into sea urchin eggs blocks cytokinesis and clearly showed the importance of actinfilaments in cell division. Calcium may trigger contraction, via activation of MLCK, and phosphorylation by p34^{cdc2} kinase may regulate the timing of cytokinesis. Initially, the ring contracts with constant volume, with approximately twenty filaments in each actin bundle, which may shorten by disassembly, but the number of actin filaments in the ring declines during the later stages of cytokinesis.(381)

3 B ii (b) Belt Desmosomes

Epithelial tissue cells are held to each other by adherens junctions to enable them to function as a structural unit. In non epithelial cells, these are punctate or streak like, but in epithelial cells they form a continuous adhesion belt, the zona adherens or the belt desmosome around the apical end of the cell. A continuous circular bundle of actin, similar in structure to the contractile ring, is tightly associated with the adherens junctions: actin filaments in one cell are connected to those in adjacent cells through the adherens junctions, and they have an important role in maintaining epithelial sheet integrity and increasing its strength. The contractile activity of the actin bundle, via its interaction with myosin at the apex of the epithelial layer, is thought to be directly involved with tissue morphogenesis since polar changes in the cell shape, caused by contractions in the cytoskeleton produce folding of the epithelial sheet, to form invaginations seen at the first stage of the development of structures, such as the neural tube, and at gastrulation.(39, 136, 56, 266)

3 B ii (c) Actin Bundles in Wound Healing

In a large epithelial wound, lamellipodia protrude from cells at the wound edges and crawl over exposed connective tissues. In small epithelial wounds, however, and skin wounds in embryos, the circumference narrows like an iris, the edges appear to be drawn in as if by a purse string and the epidermis at the edge of the wound appears smooth, as if under

circumferential tension. Within five minutes, the borders of the wound begin to sequester contractile proteins, actin filaments, myosin II, tropomyosin, villin and a tight junction protein, ZO-1. The actin filaments form a contractile cable which appears to run around the wound margin since it is continuous from cell to cell (presumably via adherens junctions) and is confined to a single row of basal cells at the free edge of the epidermis. These filaments are present until the wound is closed. Wounding results in an increase in the number of gap junctions in cells bordering the wound, and therefore an increase in junctional communication between adjacent cells, leading in turn to the coordination of the actin cable contraction. No change in the thickness of the actin cable is observed as it shortens during wound closure, nor is there any observed change in the average shape of cells through which the cable runs, suggesting that cells leave the marginal row as the wound heals. The formation and contraction of actin cable may be triggered by changes in cell tension on wounding and by influx of calcium²⁺. (287,14)

3 C ACTIN BUNDLES AND ANCHORAGE DEPENDENT GROWTH

Changes in cell shape and cytoarchitecture are important in determining cellular metabolism, regulation of growth and expression of genes related to terminal differentiation and stress fibres which in part determine cell shape, are often found in normal tissue associated cells grown in culture (25, 55).

Normal adherent cells which would form a solid tissue *in vivo*, rarely divide in suspension culture. This relationship between cell spreading and proliferation is known as anchorage dependence of growth or division (25, 55, 23, 22). By plating cells on a substrate of variable adhesiveness (produced by different concentrations of poly (2-hydroxyethylmethacrylate) (polyHEMA)) it has been demonstrated that a strong attachment and a spread cell shape were required for growth and proliferation, as flattened cells divide more frequently than rounded cells (23). However, a direct correlation between the formation of stress fibres and growth control is not always present since a number of cell lines do not require stress fibres for growth and proliferation, including, for example, HeLa PTK - 1 and SP2 cells. The response of cells to several growth factors is also linked to cell shape since cells in suspension culture do not respond to growth factors and, for example,

the growth of normal cells is enhanced by increasing the concentration of serum as they become well spread. (448) Both growth and division may be regulated by signals received via an organised cytoskeleton (21). Attachment and adhesion (via few cell: substrate contacts) and cell spreading (via more extensive contacts) and therefore cell shape appear to be crucial elements of growth control (25). Both require integration of the cortical actin cytoskeleton and stress fibres since stress fibres maintain the flattened, well spread shape of attached adherent cells and the cortical cytoskeleton attaches to transmembrane receptors. Cells in suspension culture are blocked in G1 stage of the cell cycle (25) and protein, RNA and DNA synthesis are arrested with protein synthesis declining gradually as mRNAs are withdrawn from translation (23,448,108,18). Recovery of macromolecular metabolism is switched on by contact with the substrate and changes in cell shape, as contact and respreading increase (25). On reattachment, protein synthesis recovers rapidly, using pre-existing mRNA which in suspended cells is reversibly modified into a stable but untranslatable form with an extended half life (108). Rapid recovery of protein synthesis is triggered by the establishment of only a few contact points between the membrane and the substrate and does not require cell spreading (23, 448) However DNA and RNA synthesis are profoundly affected by cell shape and require extensive cell spreading which reinstates progress through the cell cycle with a direct correlation between cell shape and DNA synthesis, so that even small, subtle changes in cell spreading have a large effect on DNA synthesis (23) Given the role of the cytoskeleton in determining cell shape and cell attachment, control of cell growth and division must be coupled to cytoskeleton organization. For example, alteration of microfilament and microtubule organization dramatically affects initiation of DNA synthesis and the important role of stress fibres in the control of cell growth is suggested by the observation that the synthesis of actin is specifically inhibited relative to other proteins in suspension culture (25). On reattachment there is a marked superinduction in the synthesis of actin and about 8 hours after replating when protein synthesis has recovered to control levels, it accounts for 25% of total protein and requires new synthesis of mRNA in addition to pre-existing mRNA.(108) This overproduction of actin may represent specific gene activation in response to suspension culture conditions and it is possible that altered physical configurations may induce a

metabolic response which affects cell morphology (17). Cell shape is determined to a large extent by the organization of the cytoskeleton (21). Changes in actin synthesis in suspension/reattachment studies parallel the changes observed in actin synthesis during stimulation of quiescent cells by growth factors (PDGF FGF) which suggests a link between cytoskeleton organization, cell shape changes, gene expression and growth control (25). Expression of genes encoding several cytoskeletal proteins (vimentin, cytokeratins and vinculin) are sensitive to the degree of cell : cell and cell : substrate contact. Vinculin synthesis correlates with the degree of cell spreading and a feedback control mechanism is regulated by alterations in adhesiveness of the substrate with a five fold decrease in vinculin synthesis in nonadherent compared to spread cells (448). Vimentin synthesis is maximal with increased cell to substrate contact and is therefore regulated by cell shape and substrate interactions, whereas cytokeratin expression is maximal with increased cell to cell contact and is regulated independently of vimentin synthesis (25, 448).

The mechanism by which cell shape is transduced to a controlling signal for macromolecular metabolism is not known, but adhesion and spreading seem to be required for the transmission of mitogenic signals to the nucleus (23) Fibroblasts respond to cell shape by regulating growth, while other cell types show a different response. Expression of a differentiation related phenotype is also dependent on cell shape and shape modulation of epithelials which have only a few stress fibres and chondrocytes radically changes their spectrum of synthesized proteins (448). It is possible that changes in cell shape and cell spreading mediated by the cytoskeleton, leading to the regulation of growth, cell differentiation and motility regulate macromolecular metabolism and have a role in cell behaviour in the formation of metazoan tissue (23). The loss of growth control [anchorage dependence] in tumour cells, a central feature of cell malignancy is associated with a permanent decrease in cell attachment, loss of focal contacts, stress fibres and the acquisition of rounded morphology (484, 25). In suspension cultured and transformed cells the structural cytoskeletal networks which normally transduce cell shape/surface contact signals to cell metabolism are disorganized (484, 25). For example, in 3T6 cells, where anchorage dependence and division is lost/limited and cells grow in an uncontrolled manner in the absence of substrate contact, actin synthesis is inhibited to a lesser degree

than that of normal 3T3 cells (484). Growth of melanoma cells *in vitro* in a spherical configuration induced a marked increase in metastatic capability of cells *in vivo*, in contrast to growth as a monolayer in a flat configuration suggesting that a central role for cell shape is present in the modulation of metastatic capability. Loss of shape responsive control is related to tumour progression (363, 448).

3 D CELL ADHESION TO THE SUBSTRATE

A variety of proteins anchor the actin cytoskeleton to the plasma membrane (415) at sites called adhesion plaques. These are defined regions where a cascade of structural and regulatory proteins anchor actin filaments to the plasma membrane and, via transmembrane receptors, to the extracellular matrix. Adhesion plaques were first identified in cultured cells by interference reflection microscopy as dark, elongate or arrowhead shaped areas on the ventral surface of the plasma membrane, where the cell surface was closely opposed to the substrate (269,55,54,165).

Adhesion plaques are associated with reduced cell locomotion and are therefore prominent in stationary cells and absent in locomoting cells (54)(although small areas of contact at the leading edge of the cell are required for traction during locomotion (269)). They are responsible for cell attachment to the substrate and may regulate the assembly and disassembly of the actin cytoskeleton (224,54).

In mediating cell adhesion and cytoskeleton organization, adhesion plaques play an important role in many fundamental processes, including the maintenance of cell shape, morphogenesis, cell polarization, cell motility, development and the regulation of growth and differentiation. They enable tension to be transmitted across the plasma membrane via transmembrane receptors to the extracellular matrix and are found to be sites of signal transduction (394).

Adhesion plaques (via the integrins clustered there) act as mechanoreceptors and transmit mechanical signals from the substrate to the cytoskeleton. This recognition of and response to mechanical stimuli are critical to growth and cell function including morphogenesis, tissue repair, tissue integrity and organization, cell development, migration and inflammations. For example, it has been shown that stretching a flexible extracellular

matrix substrate induces cytoskeletal reorganization and biochemical changes in adherent cells. In addition to growth factor stimulation, tissue associated cells must adhere to a substrate that can resist tension, and spread before they can enter the S phase of the cell cycle therefore, nuclear architecture and function, such as DNA synthesis and transcription, may be regulated by tension generated by cell spreading. (394, 468,165,201)

In addition, it has been suggested that the integrin cytoskeleton linkages are part of a group of molecular elements that are organized according to the tensegrity building concept of Buckminster Fuller, in which all three elements of the cytoskeleton (microfilaments, microtubules and intermediate filaments) are integrated to resist deformation in response to stress and transduce mechanical signals throughout the entire cell more rapidly than a diffusion based signal system.(178,468)

Although they are prominent in cells *in vitro*, adhesion plaques do not appear to be a tissue culture artefact since structurally and functionally similar regions are found *in vivo* where cells interact with and transmit tension to the extra cellular matrix. These regions include dense plaques in smooth muscle, myotendinous junctions in skeletal muscle, basal surfaces of epithelial cells where they attach to the basement membrane, and belt desmosomes in epithelial cells. In addition, proteins found in adhesion plaques in cultured fibroblasts are abundant in non-cultured cells such as platelets where talin redistributes from diffuse cytoplasmic distribution to the cortex on activation (54, 13).

3 D i Adhesion Plaque Proteins

Although many components of adhesion plaques have been characterized, the mode and stoichiometry of their assembly is not yet fully understood and the interactions between proteins appear to be very complex (193). Models of adhesion plaque composition and organization are largely based on immunofluorescence colocalization studies, light microscope and electron microscope studies, and *in vitro* binding studies (137,445) such as equilibrium column chromatography, which demonstrate, for example, an interaction between talin and integrin (189), gel overlays with radiolabelled proteins (54, 326) solid phase binding (336) and solution binding assays (54). Integrins (heterodimeric extracellular matrix) receptors span the plasma membrane (423) and bind extracellular molecules and the

short cytoplasmic domain of integrin β_1 subunit binds fibulin (9) talin (326) and α actinin (336). Talin also binds vinculin, nucleates actin assembly and at high protein concentrations may associate into homodimers (445). Vinculin binds paxillin (444), α actinin and may oligomerize while zyxin and actin both bind to α actinin which also bundles actin filaments. Tensin, which may bind to vinculin, caps the barbed ends of actin filaments, and contains an SH₂ domain which mediates its interaction with adhesion plaque proteins that contain phosphotyrosine residues. As SH₂ domains are found in several proteins involved in signal transduction, a regulatory function in adhesion plaque organization has been proposed for tensin. (445, 269) In addition, at least one member of the gene family that includes radixin, ezrin and moesin (proteins that are thought to mediate membrane / cytoskeletal association) tenuin, and VASP (vasodilator stimulated phosphoprotein) which binds to actin in sedimentation assays are localized to adhesion plaques (269, 445). An additional level of complexity is added by the observations that α actinin, vinculin and talin can all bind to acidic phospholipids (269). These data have been used to propose possible chains of attachment between actin and the extracellular matrix (54, 326). One model proposes actin : α actinin : vinculin : talin : integrin (54), while another suggests that integrins connect to actin via two linkages, the first being talin : vinculin : tensin : actin, and the second direct attachment of integrin to actin by α actinin or talin (394). Other proteins might aid in or regulate these, and/or other parallel sets of links between stress fibres and the extracellular matrix (326). For example, while paxillin binds to vinculin, interactions with other structural proteins have not yet been found, ruling out, at least at present, a structural role for paxillin. However, its low abundance at adhesion plaques in comparison to talin and vinculin, its association with the SH₃ domain of pp60^{src}, and its tyrosine phosphorylation to a high stoichiometry (30 %) by pp60^{src} suggest that it may have a regulatory function. Zyxin is also present in relatively low abundance, and therefore is proposed to have a regulatory rather than a structural role. (443,444) In addition, the presence of a number of known regulatory proteins in adhesion plaques , including a protein kinase C isoform, ppFAK¹²⁵, and pp60^{src}, and a calcium activated protease, calpain II, which cleaves talin in the presence of calcium suggests that they are likely to regulate adhesion plaque

organization during cell processes such as cell locomotion and division, which involve coordinated cycle of attachment and release.(269, 443, 55,445)

Current models are still unlikely to be complete. The identification of new proteins, biochemical characterization of the mechanisms of various intermolecular interactions and the assignment of a function to each component will help in understanding of the structure and regulation of adhesion plaques (137,326,54). Procedures to isolate adhesion plaques with their structural integrity preserved will assist in this study (440).

The *in vivo* functions of adhesion plaque proteins are being tested by alterations of the amount of individual proteins. For example, microinjection of talin or vinculin antibodies disrupts both stress fibres and adhesion plaques(269) and transfection of NIH 3T3 cells with vinculin anti sense mRNA leads to rounded, poorly spread phenotype, smaller and fewer adhesion plaques and increased motility (114). Conversely, over-expression of either vinculin or α actinin in transformed cells inhibits motility and suppresses tumorigenicity, and these changes in cell behaviour are paralleled by increases in the size and stability of adhesion plaques in these cells (377, 145). These observations, that adhesion plaque integrity and function can be dramatically changed by the alteration of a single component, suggest that these proteins are not redundant, but obligate, with each one making a significant contribution to an 'obligate' structural and regulatory protein cascade.

3 D ii Adhesion Plaque Assembly

Adhesion plaque assembly appears to be initiated by the binding of extracellular matrix components (fibronectin, vitronectin or heparan sulphate proteoglycan) to integrins, possibly by inducing a conformational change in the cytoplasmic domain of integrin (thus exposing one or more cytoskeletal binding sites) or by phosphorylation. Ligand binding to their extracellular domain induces the aggregation and immobilization of integrins (269,309) and this stimulates the accumulation of soluble talin to the site of integrin extracellular matrix contact. Talin is localised in the ruffling membrane of fibroblasts, and at the tips of filopodia, which are adhesion plaque precursors. Talins direct interaction with actin may be an early event in adhesion plaque assembly with α actinin, vinculin and other components then being recruited to the developing adhesion plaques and stabilising them. Further actin assembly is nucleated (55, 265). A cooperative model for adhesion plaque

assembly from soluble components has been suggested (114). The low affinity interactions between, for example, talin and integrin would appear to be insufficient to maintain a link between the cytoskeleton and integrins in view of the tension generated across the membrane at adhesion plaques (189). However, the high tensile strength generated across these adhesion sites may be made possible by the integration of multiple low affinity interactions that occur between the numerous components (443)

Finally, the adhesion plaque is a dynamic structure (net movement of an entire adhesion plaque has been observed (54) and some proteins, for example vinculin and talin, exchange between cytoplasmic, soluble and membrane pools, even after the adhesion plaque is established. This dynamic equilibrium may affect the size and stability of the adhesion plaque and after initial formation, some will disassemble while others enlarge and nucleate associated stress fibres (54). Disruption of integrin : substrate interactions by the RDG peptide causes dissociation of cytoskeletal proteins, such as vinculin and α actinin from the adhesion plaques and cell detachment (269, 309)

3 D iii Regulation of Adhesion Plaque Assembly

Although regulation of adhesion plaque stability is important in controlling processes such as cell motility and morphology, this process is poorly understood .

Changes in protein phosphorylation are very likely to be important in regulating the structure and function of adhesion plaques (443, 269), as kinases including protein kinase C and pp125^{FAK}, and pp60^{v-src} in transformed cells, are concentrated at sites of cell to substrate adhesion, and tyrosine phosphorylation of paxillin, tensin and pp125^{FAK} accompanies both v-src mediated transformation (β_1 integrin, talin and vinculin) and cell adhesion to extracellular matrix. The phosphorylation of pp125^{FAK} activates its kinase activity and the resulting phosphorylation of paxillin by pp125^{FAK} may enable the recruitment of SH₂ containing proteins such as tensin to adhesion plaques. Herbimycin A, an inhibitor of cellular protein kinases inhibits the formation of adhesion plaques, suggesting that tyrosine phosphorylation may be an important event accompanying attachment rather than disruption of cell : extracellular matrix interactions (269). Phosphorylation events in the adhesion plaque appear to be triggered by the adhesion of

integrins to extracellular matrix components, which stimulates their clustering, as it has been shown that protein kinase phosphorylation of a limited number of proteins occurs shortly after fibroblast attachment to laminin, but not polylysine, which does not induce integrin clustering. As integrins do not have intrinsic kinase activity, this signalling must be indirect, and it is not clear at present, how clustering of integrins initiates phosphorylation of, for example, pp125^{FAK}. It may bring together multiple low affinity binding sites to create a high affinity binding site so that signalling proteins can then bind and be activated. For example, clustering may allow two non receptor kinases to be in close proximity, enabling cross phosphorylation. An additional factor in the regulation of adhesion plaque may be introduced by the rise in calcium levels that accompany integrin : ligand interaction, which might affect actin cross linking by a actinin, and activate calpain II, which proteolyzes talin (384, 394,309, 440,55).

3 D iv Adhesion Plaques as Sites of Signal Transduction

In addition to their structural function, integrins may have a role in cell signalling as ligand binding induces a number of responses inside the cell, such as changes in pH by interaction with the Na⁺ - H⁺ antiporter , calcium transients, increases in tyrosine phosphorylation, inositol lipid turnover and changes in gene expression (394, 165) using pp125^{FAK} as an intermediate signalling protein (384)

Integrins may also act in synergy with growth factors such as PDGF and therefore link adhesion and cell spreading to control growth and division (394, 193). For example, PDGF can induce lipid hydrolysis in adherent but not suspension cultured cells and the failure of phosphorylated PLC γ to hydrolyse PIP₂ in suspended cells appears to be due to an effect of integrin mediated adhesion on PIP₂ synthesis. The amount of PIP₂ decreases in detached cells and increases on their reattachment and these differences in levels appears to be due to different rates of synthesis. Integrin : fibronectin mediated cell adhesion triggers the activation of PIP5 kinase, which phosphorylates PIP to PIP₂. PIP₂ can then be hydrolyzed by phospholipase C γ , which is activated by PDGF/receptor binding, to second messengers DAG and IP₃ which activate protein kinase C and induce calcium release respectively which leads to alterations in gene expression (394,55). The synthesis of PIP₂

in response to integrin mediated adhesion may also mediate some of the observed effects of adhesion on the cytoskeleton by affecting actin polymerization at sites of substrate contact via the interaction of PIP₂ with proteins such as profilin and gelsolin (394).

3 E NUCLEAR ACTIN

Actin has been found in the interphase nuclei of various cells, and for example in the *Xenopus* oocyte nucleus, high concentrations (3-4 mg /ml) of mostly soluble, but polymerization competent, β and γ actin exists in the nucleocytoplasmic exchange pool (462,385,8). Some actin also exists as short polymers (385). A number of actin binding proteins are also present in the nucleus and these include myosin (333), cofilin (319), ADF, HSP 90/100, 30 kDa *Acanthamoeba* actin binding protein (364), nuclear actin binding protein (8) CAP Z and gCAP 39/mbh-1(333). Nuclear actin filaments are likely to be regulated by these proteins. Proteins can enter the nucleus by passive diffusion, or by active transport across a nuclear envelope channel after targeting with a nuclear localization site, or binding to a chaperone which contains a nuclear localization site (333). Phosphorylation may be an important signal for nuclear localization and nuclear gCAP39 is highly phosphorylated(333). In addition to its proposed role in regulation of the nuclear matrix structure/architecture , at least part of the nuclear actin appears to be involved in specific nuclear functions, such as DNA replication, transcription and RNA processing . Injection of anti-actin antibodies, or anti-fragmin antibodies into *Xenopus* oocytes, blocks transcription by RNA polymerase II, loop retraction and chromosome condensation (462,385) and α and β actin is required for accurate transcription by RNA polymerase II (385). Actin filaments have been observed to interact with pre mRNAs in the nuclear matrix of mouse cells (462, 385).

III

1. ACTIN ASSOCIATED PROTEINS

Actin organization is controlled by a large number (100 are so far known) (430,297) of actin associated proteins which, by their coordinated action (up to 40 actin binding proteins may be found within a single cell) (432) regulate the time, place and extent of actin assembly, G : F actin ratio, actin architecture/geometry and provide both the fine control and driving force required for the variety of cellular events in which the actin cytoskeleton participates (347,388). Different actin assemblies, each with specific actin cross-linking proteins, can co-exist and function differently within the same cell at the same time, for example within the hair cells of the cochlea (stereocilia, cuticular plate and circumferential belt, 99,407) and in fibroblasts, at least four distinct structural domains are occupied by actin filaments, namely, stress fibres, ruffling membranes sites of filament anchorage to the plasma membrane and the contractile ring during cell division (407). Actin binding proteins act within the cell to sequester monomers and inhibit actin assembly, create nucleation sites and initiate polymerization, cap filament ends and regulate their length, sever actin filaments, and cross-link filaments into a variety of three dimensional structures from highly packed bundles of microvilli to the cross-linked meshwork of the cortex (476, 82). Motor proteins (myosins) enable the generation of force. Although actin associated proteins can be classified according to their sequence into gene families (100), they have classically been grouped by function (414, 351, 236) and I have adopted this classification in the following review of these molecules.

1 A ACTIN MONOMER BINDING PROTEINS

These proteins bind to G actin monomers and inhibit their addition to actin filaments. They therefore induce depolymerization of F actin as actin monomers dissociate from filaments to restore the steady state monomer concentration.(351)

1 A i Thymosin β 4 (FX)

Originally identified in calf thymus as a complex with actin by mobility on nondenaturing gel (375), thymosin β 4 is a 5 kDa, monomeric, actin sequestering protein that is widely

distributed in vertebrate cells. It binds to G actin monomers in a 1:1 complex that inhibits salt induced actin polymerization, but does not sever, cap actin filaments or specifically inhibit nucleation (374). Formation of an actin : thymosin β 4 complex inhibits ATP exchange by actin monomers, and thymosin β 4 has a higher affinity (50 fold) for ATP than ADP actin. Actin binding activity does not appear to be regulated by calcium, phosphorylation or PIP₂ (374). Thymosin β 4 is most abundant in platelets and neutrophils and macrophages and is also present in high levels in fibroblasts. Its concentration and affinity for actin suggests that the bulk of the G actin pool is complexed with thymosin β 4 in quiescent / resting platelets, where the barbed end of actin filaments are capped (430, 374). Stimulation of platelets by thrombin leads to a rapid decrease in the level of actin : thymosin β 4 complex, and a parallel increase in the concentration of F actin filaments. The release of actin from the complex may be regulated in part by the higher affinity of newly uncapped barbed ends than of thymosin β 4 for free G actin (374, 475).

Nine thymosin β s have been identified so far, six of which bind to actin and of these, thymosin β 4 and thymosin β 10 function primarily as G actin buffers. Regulation of synthesis has been shown for thymosin β 10 in brain development and for thymosin β 4 after stimulation in several cell types. All vertebrates studied express one or two thymosin β isoforms, and an invertebrate isoform has been identified in sea urchins.(314)

All thymosin β s contain a hexapeptide (LKKTET) that inhibits actin polymerization. This region shares homology with several other actin binding proteins, for example, actobindin, tropomyosin, α actinin and myosin heavy chain.(453)

1 A ii Actobindin

Actobindin is a 9.7kDa protein found in *A. castellanii* . Its amino acid sequence has been determined and reveals a repeated segment of 33 amino acids, which are the actin binding domains. These contain a hexapeptide (LKHAET) which is common to thymosin β 4, α actinin, myosin heavy chain and tropomyosin and is known to bind actin.(453)

Although actobindin sequesters actin monomers, it binds G actin with too low an affinity to account for its potent inhibition of polymerization. The presence of two actin binding sites and marked delays in the onset of polymerization, suggest that actobindin binds tightly but

reversibly to actin dimers and oligomers, the early intermediate stages in actin polymerization, and disaggregates them, thus preventing them from nucleating actin elongation. Actobindin does not sever actin filaments, nor reduce the rate of elongation of pre-existing filaments.(240, 415)

1 A iii Profilin

Profilins are small (12 - 15kDa) basic, globular proteins that were first purified from spleen. They are among the most abundant cytoplasmic proteins, present at a concentration of 40 - 100 μM and are conserved from yeast and *D discoides* to humans (271). They are found in all eukaryotic cells examined, including plants, fungi and mammals. Isoforms of differing pI and Mr are found in human platelets and a wide range of other tissue and cell types, such as skeletal muscle, spleen, macrophages and brain (351). Many species have multiple isoforms. Interestingly, smooth muscle profilin inhibits polymerization of muscle actin more effectively than thymus profilin, suggesting that the profilins have tissue specificities (271). Profilin can act as a sequestering protein, but is also thought to act catalytically to stimulate the exchange of nucleotide and divalent cation, possibly by inducing a conformational change in actin, that opens the nucleotide cleft (an increase of fluorescence of pyrene labelled actin is seen on complexing with profilin)(351) This can occur even in the presence of thymosin β_4 , which binds to actin and inhibits nucleotide exchange (430, 271) because of the high on/off rate of profilin:actin binding (147). In conditions of rapid filament reorganization where there are large amounts of ADP actin and an excess ATP over ADP, profilin may promote actin polymerization. (430) Profilin is concentrated at the cytoplasmic membrane interface and therefore may have a major role in regulating actin assembly at the leading edge of the cytoplasm, for example in pseudopodia and lamellipodia formation. Catalysis of nucleotide exchange by the high concentration of profilin at the tail end of *Listeria* is suggested to facilitate the assembly of actin filaments that drives the bacteria through the host cytoplasm (432)

By low affinity binding and capping of barbed filament ends profilin only weakly inhibits elongation at that end. Inhibition of elongation and nucleation at the pointed end of actin filaments is much greater. In some circumstances profilin can lower the critical

concentration at the barbed end(430) and favour the elongation of preformed filaments (147).

The relatively low affinity of profilin for actin, and the two to six fold molar excess of G actin over profilin (in quiescent cells) suggest that profilin alone cannot account for the high concentration of G actin in the cytoplasm (409,430).

Profilin may link receptor mediated transmembrane signalling and microfilament based motility. In platelets profilin binds to clusters of 4 -5 molecules of PIP and PIP₂ with greater affinity than actin so that binding to polyphosphoinositides disassociates profilin from the profilactin complex and in quiescent platelets, most of the profilin is associated with the plasma membrane (148). Stimulation of the cell by, for example, EGF binding to its receptor, results in phosphorylation of receptor itself and PLC γ 1 by EGF receptor tyrosine kinase activity. Phosphorylated PLC γ 1 is able to hydrolyse PIP₂ leading to the release of the second messengers, IP₃ and DAG, and profilin which in the presence of ATP can promote the rapid F actin polymerization seen in stimulated platelets (148). Profilin may also interact with the Ras pathway in the yeast *S. cerevisiae*, as overexpression of profilin has been shown to restore a normal morphology in yeast cells with a defect in an effector of Ras (430, 271, 464).

1 A iv DNase I

DNase I is found in vertebrates as a single polypeptide chain of 29 kDa. It binds to actin monomers near the ATP binding cleft and either directly or via conformational change in the actin monomer inhibits nucleotide exchange. DNase I-bound actin cannot polymerize, so the rate and extent of polymerization are inhibited (351, 414) DNase I can bind weakly to the pointed end of the filament and prevent elongation (474). Binding to actin inactivates its enzyme activity (414) The *in vivo* function, if any, of DNase I : actin binding is unclear (6).

1 A v Actin Binding Protein 50 (ABP 50)

ABP 50 was originally isolated from *D. discoidea* and is an abundant (1% of total protein) basic globular actin associated protein of 50 kDa. It was later identified as EF 1 α , a protein

involved in eukaryotic protein synthesis. The reversible association of ABP50 with the cytoskeleton following stimulation of cells may tether the protein synthetic machinery to the actin cytoskeleton, providing a mechanism for the spatial and temporal regulation of protein synthesis (495, 292).

In resting *D. discoides* amoebae, 90% of ABP 50 is found in the cytosol in 1 : 1 complexes of G actin monomers. The enzymatic activity of EF 1 α is inhibited by G actin binding (495) (which may inhibit EF 1 α nucleotide exchange). The remainder is associated with filamentous actin. During chemotactic stimulation, ABP 50 is increasingly associated with cortical regions of the cytoskeleton especially newly formed extensions such as filopodia (495,170). The enzymatic activity of the protein is restored on binding to filamentous actin, although there appears to be no difference between the affinity of ABP 50 for G or F actin. (170). EF1 α complexes with GTP and aminoacyl tRNA and catalyzes the codon dependent binding of aminoacyl tRNA to the A site of ribosomal particles in the peptide elongation phase of protein synthesis.

Consistent with its localization in areas of the cell which contain filamentous actin bundles, ABP 50 can cross-link actin filaments into bundles. This activity is calcium insensitive and the pattern of filament packing within these bundles can vary and ABP 50 can cross-link neighbouring filaments that are rotated at right angles relative to each other (170).

ABP 50 shows some sequence homology with depactin, actin and actobindin (495)

1 A vi Vitamin D binding protein: (DBP/Gc. Gc protein)

Vitamin D binding protein (55kDa) is a glycoprotein found in the plasma of vertebrates, and can bind with high affinity to monomeric actin in a 1:1 complex. *In vivo*, its interaction with actin is thought to result from the leakage of actin from necrotic or apoptotic cells. With brevin, this is a mechanism whereby actin filament fragments released from cells can be depolymerized and sequestered (as monomers are unable to polymerize) . The presence of filamentous actin in blood would increase its viscosity, impair blood flow and may cause tissue damage (351).

1 B SMALL SEVERING PROTEINS

This group of proteins, isolated from vertebrate and invertebrate sources, probably sever actin filaments by sequestration of actin monomers from within the filament, an action which has been described as 'nibbling' and do not cap severed filaments (273). They bind filaments with a lower affinity than gelsolin, and are therefore less efficient severers, than the gelsolin family of capping/severing proteins (273). They may promote actin polymerization by generating more filament ends. The family includes depactin (from star fish oocytes), ADP, actophorin (from *A castellanii*), cofilin, destrin (from vertebrate kidney) and ADF (from chicken brain) (273,11)

1 B i Depactin/Actophorin/ADP (15 - 20 kDa)

Depactin (17.6 kDa) (ADP / actophorin) forms a tight, non polymerizable 1 : 1 complex with actin that does not nucleate polymerization (273) and depactin may slow the initial rate of polymerization. Actin can be released from the 1 : 1 complex when required by the cell for polymerization into filaments, but the factors that dissociate the complex have not yet been identified, although polyphosphoinositides may be involved as depactin binds PIP₂ and PIP, and, *in vitro*, myosin can dissociate the complex in the absence of ATP, possibly by competition for binding sites on actin molecules. Actophorin and profilin compete for G actin, and actophorin has a higher affinity for G actin (273). Depactin binds (weakly) to actin filaments in a 1 : 1 ratio to actin monomers within a filament and destabilizing it by extracting the monomer which leads to filament breakage. This is seen *in vitro* as a decrease

in the viscosity of actin (273, 414). Depactin is not regulated by calcium or pH, but the severing action can be inhibited by phalloidin (which might prevent the interaction of depactin/actophorin with actin subunits) or inorganic phosphate, which restores ADP actin within the filament to ADPP_i which has a more resistant conformation (273). The binding site for actophorin on filamentous actin may be exposed by the movement of filaments, since a higher frequency of severing is seen at preexisting bends in actin filaments. If depactin is present during salt induced polymerization of actin, the rate of polymerization is initially slow because of the sequestration of G actin by depactin and then accelerates and rapidly reaches completion as the severing activity of depactin generates seeds for polymerization. This increase in elongation rate by virtue of filament severing activity has also been shown for actophorin which does not appear to cap filament ends. (272, 273). Actophorin/depactin shows some homology with ADF, but amino acid sequence homology with other actin binding proteins is unknown (236).

1 B ii Actin Depolymerizing Factor (ADF) (Destrin)

ADF (18.5 kDa) is a phosphoinositide binding, actin associated protein, originally isolated from chick brain (11), and is found throughout the cytoplasm in cells from vertebrates with higher concentrations along the leading edge of ruffled membranes in cultured fibroblasts and growth cones of neurones (3, 170). Amino acid sequence of chick brain ADF is closely related (73% identity) to porcine brain cofilin but they are distinct proteins (3). A sequence of amino acids DAIKKK found in ADF as well as in tropomyosin cofilin and depactin may represent an actin binding sequence. ADF also contains a sequence similar to the nuclear transport signal of SV40 large T antigen (as does cofilin) and is found in the nucleus (3). ADF induces rapid depolymerization of filaments through a weak (calcium independent and pH dependent) F actin severing activity, and sequesters monomers in a 1 : 1 complex. It does not cosediment with filamentous actin *in vitro* assays, nor cap filament ends (3, 170). The expression of ADF is developmentally regulated in muscle, and increases in F actin and myofibril assembly correlate with a decline in the levels of ADF. It may have a role in the delivery of actin to the site of filament assembly (11). Recently, an isoform of ADF

that is phosphorylated on serine residues, has been identified which does not bind to G actin, depolymerize actin or affect the rate or extent of actin assembly (306).

1 B iii Cofilin

Cofilin (21 kDa) is a phosphoinositide sensitive (498) actin modulating protein that can bind stoichiometrically to both G actin and the sides of F actin filaments (414). It was originally isolated from porcine brain (277) and is widely distributed in vertebrate cells and tissues (170). In cultured cells exposed to stress, such as heat shock the formation of actin rods is induced and cofilin is specifically associated with these paracrystalline actin structures (319). Cofilin shares sequence homology with the nuclear localization signal sequence of SV40 large T antigen, which, regulated by phosphorylation, mediates movement of cofilin into the nucleus. This cytoplasmic/nuclear shuttling of cofilin may provide an actin pool for the nucleus (319, 459).

Cofilin regulates actin polymerization and depolymerization in a pH dependent manner (500). At high pH (8.2) the ability of cofilin to depolymerize F actin is strong and can go to completion. However, near neutral pH (7) cofilin binds 1 : 1 with monomers in F actin, shortens the average filament length by inducing partial depolymerization, and increases the steady state concentration of G actin to a limited extent. It also binds G actin at neutral pH at a 1 : 1 molar ratio . At pH 6.6, cofilin rapidly polymerizes actin and binds to F actin (500). The rise in pH that occurs in cells stimulated by growth factors and tumour promoters is necessary for initiation of the DNA synthesis and correlates with disruption of the actin cytoskeleton (500). Cofilin does not nucleate actin polymerization nor cap filaments (318), but cofilin can decrease the rate of ATP exchange on G actin (320). The interaction of cofilin with F actin blocks tropomyosin binding and inhibits actin : myosin interactions (318) Actin : cofilin interactions are inhibited specifically by PIP₂, PIP and PI, and phosphorylation of cofilin in cultured cells may also inhibit its interaction with actin (498).

Cofilin is conserved in evolution. A yeast cofilin homologue is involved in the regulation of actin assembly in the cortical cytoskeleton and is important for polarized growth in budding yeast cells (303).

1 C CAPPING PROTEINS

Capping proteins associate with the ends of actin filaments (usually the barbed ends) inhibiting further elongation, annealing (476) and dissociation of monomers (130, 351, 414) at that end. Capped filaments can also be the result of the nucleating or severing activity of some of these proteins (for example gelsolin). Capping proteins do not generally effect depolymerization, but regulate actin filament length and number by their activity, that is, they shorten filaments by severing, thus influencing the nucleation and elongation steps of filament assembly (82) and they reduce the lag phase, by nucleation of actin filament assembly and by the production of more filament ends. This can affect overall actin polymerization (which is favoured by increased filament numbers) and the structure of filament networks in, for example, the cortex. Alteration of the filament number and length, especially in the cortex, can affect both cell morphology and cell motility. Calcium regulates the activity of some of these proteins. Yeast Act 2, a divergent actin is also thought to act as a capping protein (257), and ADP ribosylated actin, (modified by bacterial toxins), will cap the barbed ends of actin filaments (6). Few pointed ends cappers have been identified in non-muscle cells, but it is assumed that one must be associated with the pointed end of thin filaments in skeletal muscle. Tropomyosin associates with the pointed ends of these filament and prevents their depolymerization, but not their assembly (49). A pointed end capper is presumed to control polymerization and depolymerization of actin filaments that form a "comet tail" of filaments that drives *Listeria* through the host cell by the addition of monomers to the barbed end of the filament (432).

Simple Barbed End Capping Proteins

1 C i Cap Z/Capactin

These heterodimeric capping proteins are a family of actin binding proteins found in all eukaryotes examined from yeasts to mammals (203), that bind to two terminal actin subunits and cap the barbed ends of actin filaments (170), with no difference in ability to bind to ADP actin, ADPP_iactin or ATPactin terminal subunits (57). They can nucleate the polymerization of actin monomers by binding two monomers at their barbed poles or by

stabilizing spontaneously formed intermediate oligomers but they do not sever F actin (130, 474, 57, 80). The activity of these capping proteins is not regulated by calcium, but is inhibited by ionic phospholipids such as PIP₂ which bind to the α subunit (474)

The Cap Z proteins are heterodimers composed of α (32 - 36 kDa) and β (28 - 32 kDa) subunits (474). Cap Z was so called because it was originally discovered in the Z line of skeletal muscle (170) This location, together with their *in vitro* barbed end capping and nucleating activity suggest that CapZ may nucleate filament polymerization during myofibril assembly and thus define the polarity of actin filaments and in mature muscle, it may mediate the attachment of the barbed ends to the Z line. In non-muscle cells, CapZ may attach barbed filaments to the membrane or to other intracellular structures and, as it both caps filaments and nucleates filament assembly, may play a role in the regulation of the isotropic cortical actin network, and hence the mechanical properties of the cytoplasm. Cap Z might for example, inhibit actin network formation by eliminating end:side interactions between actin filaments (80, 170).

A role for CapZ in the regulation and organization of actin cytoskeleton is also suggested by the observation that deletions of the gene encoding CapZ homologue β subunits in *S. cerevisiae* leads to an altered cytoskeleton. In wild type yeast, this capping protein colocalizes with actin at the tips of growing buds and is not found in actin cables nor in contractile rings (474).

Interestingly, Cap Z is found mainly in the nucleus in non muscle cells and *Xenopus* oocytes where it has been suggested that it may link chromosome movements with actin based activity(170).

1 C ii Radixin

Radixin is an 82kDa monomer that binds to and caps the barbed end actin filaments, inhibiting actin filament assembly . It is not regulated by calcium. Radixin is a major component of cell to cell, but not cell to substrate, adherens junctions in interphase cells and is thought to have a crucial role in the maintenance and organization of actin filament bundles in nonmuscle cells and an important role in tight end-on association of actin filaments with the plasma membrane (439). Radixin accumulates at the cleavage furrow at

the onset of furrowing during cytokinesis and because it is attached to a transmembrane glycoprotein and actin filaments, is thought to move cortical actin filaments to the site of the cleavage furrow (380). Radixin is a member of the moesin/ezrin family of proteins and has 75% identity with ezrin and weaker homology to band 4.1 and talin (379).

1 C iii Aginactin

Aginactin is a monomeric 70 kDa protein found only in agonist stimulated *D discoides* amoebae. It binds to and caps the barbed end of the actin filaments inhibiting polymerization. It neither severs filaments, nor nucleate filament assembly. *In vitro*, it inhibits the rate and final extent of actin polymerization and increases the apparent critical concentration. It also inhibits depolymerization of F actin (383).

1 C iv Insertin

Insertin is a 30 kDa protein isolated from chicken gizzard smooth muscle. It copurifies with vinculin which suggested that it has a membrane location. It binds strongly to the barbed ends of actin filaments and retards, but does not totally block, polymerization and depolymerization. It allows actin monomers to be inserted between the terminal actin subunit and the bound insertin molecules. Two insertin molecules bind cooperatively to the barbed end of the actin filaments and remain bound to the terminal subunit during polymerization .

Actin filaments linked to the cell membrane have been shown to polymerize at the attached barbed ends and it has been suggested that insertin allows polymerization and depolymerization of the plasma membrane bound filament ends *in vivo*. (372).

Insertin shows sequence homology with the region of tensin containing an SH₂ domain (474)

1 C v Cap 100

Cap 100 is a 100 kDa, monomeric actin binding protein, found in growing and developing *D. discoides* amoebae, that caps the barbed end of actin filaments, thus delaying polymerization, without severing F actin. The capping activity which is independent of

calcium is reversibly inhibited by PIP₂, as binding to PIP₂ dissociates Cap 100 from F actin. Cap 100 can nucleate actin assembly, but only at high concentrations and in conditions of low salt. At physiological salt conditions, Cap 100 delays polymerization, possibly by inactivating intermediate actin oligomers formed by spontaneous nucleation (185).

1 C vi Tensin

In vitro, tensin (170 kDa) caps the barbed ends of actin filaments and inhibits actin polymerization. *In vivo*, it is localized to adhesion plaques in fibroblasts, the Z line of striated muscle and cell : cell contacts in epithelial cells. It is likely to have a role in transmembrane linkage of actin to the extracellular matrix in the adhesion plaque and to maintain tension in actin filaments by linking them to other structures. Tensin has an SH₂ domain that enables it to bind to tyrosine phosphorylated proteins and is itself phosphorylated on tyrosine residues in pp60^{v-src} transformed cells (269)

Barbed End Capping/Severing Proteins

Severing by these proteins is nonproteolytic, but is due to the weakening of the noncovalent bonds between adjacent actin monomers in the filament (417, 297).

1 C vii gCap 39, Macrophage Capping Protein /MCP, Mbh-I

gCap 39 is a 39 kDa calcium and polyphosphoinositide regulated barbed end capping protein (170, 333). It is abundant in macrophages and is also widely distributed in other vertebrate cells. It has 48% sequence identity with gelsolin and shares structural similarities, but in contrast to gelsolin and other members of this family, it does not sever actin filaments (356). As gCap 39 is present in the actively motile regions of cells, which are sites of rapid actin filament assembly, and it is activated by calcium and inhibited by polyphosphoinositide, its primary function appears to be the capping and uncapping of actin filaments, therefore regulating cycles of polymerization and depolymerization during a variety of motile events, following agonist stimulation (356, 333). gCap 39 dissociates from actin filaments when calcium levels fall or when the level of polyphosphoinositides

increases, without there necessarily being a decrease in calcium concentration. While calcium has a reversible effect on gCap 39 actin binding, calcium dependent actin binding by gelsolin is only partially reversible (213). As gCap 39 and gelsolin are regulated differently by calcium and polyphosphoinositides, it is possible that gCap 39 can nucleate actin assembly under conditions in which gelsolin remains associated to barbed filament ends.

gCap 39 is present in the nucleus of interphase cells, redistributing throughout the cytoplasm during mitosis. Nuclear gCap 39 is more highly phosphorylated than cytoplasmic gCap 39, suggesting that phosphorylation of its nuclear targeting signal controls migration into the nucleus (333). Mbh-1 (myc basic motif homolog - 1), a gCap 39 homologue from mouse 3T3 cells, contains a c-myc oncoprotein-like DNA binding motif and a nuclear targeting signal (356) and also has both a nuclear and cytoplasmic localization. gCap 39 is therefore thought to regulate nuclear and cytoplasmic architecture via its interaction with actin (356). gCap39 may, in a manner similar to profilin, inhibit phospholipase C γ hydrolysis of PIP₂ and therefore regulate polyphosphoinositide turnover, competing with phospholipase C for PIP₂ (213, 356, 333). gCap 39 is also a minor component of plasma and appears to be actively secreted where it may be involved in the clearance of actin from the extracellular environment (213)

1 C viii Severin

Severin is an abundant 40kDa monomeric protein found in *D discoides*. It severs actin filaments, nucleates actin assembly and caps the barbed end of actin filaments in a calcium dependent manner (356). Other members of this family include fragmin, (42 kDa) from *Physarum* and a 45kDa protein from sea urchin eggs. In higher eukaryotic cells the corresponding proteins are 80 - 90 kDa gelsolin and villin. All the actin modulating properties of severin are observed in other members of the family. Actin filament binding and severing activity of severin is activated by calcium while monomer binding, F actin side binding, severing activity are inhibited by binding to PIP and PIP₂ (128, 101). The inhibitory effect of PIP₂ is increased at lower pH. As it only binds one actin monomer/molecule, it nucleates actin assembly weakly in comparison to gelsolin. Primary

structure analysis of severin, fragmin and gelsolin has revealed their modular structure and confirms their evolutionary relationship with severin containing three fold and gelsolin six fold repeats respectively (128,356,101). In a similar manner to gelsolin and fragmin, gelsolin does not dissociate from actin even when calcium is removed. Actin in fragmin : actin complexes is phosphorylated by endogenous actin kinases and this phosphorylation abolishes severins nucleating and capping activities without dissociating the complex (129,474,101)

1 C ix Fragmin 60

Fragmin 60 is a fragmin-like 60kDa protein from *Physarum* which severs F actin, caps the barbed ends of filaments and enhances actin nucleation in a calcium sensitive manner. (128).

1 C x Scinderin/Adseverin

This is a 74-79 kDa gelsolin-like actin filament severing protein found in vertebrates in secretory and neural tissue, in adrenal chromaffin cells and neuroendocrine tissue . Its activity is regulated by Ca^{2+} and inhibited by binding to PIP_2 , PI and phosphatidyl serine (170).

1 C xi Gelsolin

Gelsolin is a globular, monomeric 80 kDa protein found in vertebrates. It was first identified as a factor responsible for solating gelled extracts from macrophages. It severs actin filaments, caps their barbed ends and nucleates filament assembly and therefore it promotes the formation of large numbers of short actin filaments, capped at their (newly created) barbed ends. It is regulated by calcium, pH and PIP and PIP_2 . Gelsolin is thought to be involved *in vivo* in the restructuring and, therefore, the changes in mechanical properties of the cortical actin cytoskeleton that accompany a variety of agonist stimulated motile events, for example, the formation of dynamic structures of cell surfaces, including pseudopods and infoldings during phagocytosis, crawling types of cell locomotion, which involve gel to sol interactions and protrusive activity, and vesicle traffic through the cortical

cytoplasm (77, 297). Consistent with this suggestion is the differentiation/dedifferentiation specific control of gelsolin expression, so that gelsolin levels are increased during myeloid differentiation into highly motile macrophages (238) which suggests a relationship between gelsolin and motility); and that agonist stimulation of platelets and lymphocytes induces translocation of gelsolin towards the plasma membrane, dissociation of gelsolin actin complexes and an rapid increase in F actin concentration (170). In addition, over-expression of transfected gelsolin cDNA enhances the stimulated migration of fibroblasts, and microinjection of gelsolin fragments lacking calcium regulation, results in altered cell shape, disruption of the cytoskeletal stress fibres and increased ruffling and deformability (87, 77).

Antibody labelling has localized gelsolin to the cell periphery, the central cytoplasm and on thin actin filaments, such as microspikes, but not on stress fibres, and shown that following cell stimulation, it redistributes to the cell periphery (170).

Gelsolin : actin interactions are activated by calcium (496) so that in the presence of calcium, gelsolin will bind actin monomers in a filament orientation and promote actin polymerization by nucleating filament assembly , or bind to the side of and sever F actin filaments causing rapid shortening of filaments (297, 351, 130). Low pH also activates gelsolin to sever. The barbed ends of the fragmented actin, or gelsolin nucleated filaments remain capped by gelsolin which prevents both growth at that end and annealing of the fragments , but allows growth at the pointed end (130,297). Capping by gelsolin is not reversed by the removal of calcium but *in vitro* PIP₂ will dissociate the gelsolin cap by binding with high affinity to gelsolin, releasing actin filaments with their barbed ends free for elongation (190). PIP₂ binding also inhibits gelsolins severing activity, and profilin and gelsolin may compete for PIP₂ binding (401, 474) Gelsolin induced actin severing involves interactions of multiple domains with actin filaments, has been described as a slicing action (210,297). Primary sequence analysis reveals that gelsolin consists of six repeated homologous domains (458). Gelsolin has three distinct actin binding sites , two for G actin and one for F actin and both G and F actin binding sites are required for severing (210,470).

A point mutation in gelsolin causes an hereditary familial amyloidosis (297).

Brevin, a slightly larger isoform of gelsolin (with 25 extra amino acid residues at the N terminus) is found in plasma, where it is secreted mainly by muscle cells, and may be involved in the clearance from the blood of actin filaments released by damaged tissue (351, 237).

1 C xii Villin

Villin is a globular, monomeric protein of 95kDa which shows strict tissue specific expression and is a major structural protein associated with the actin cytoskeleton of intestinal and renal cell brush border microvilli (121). It shows homology with gelsolin (2/3 domains) but has a unique C terminal head piece which confers actin bundling activity (121). Severing, capping and nucleating activities of villin are calcium sensitive, so that at high calcium levels it induces filament depolymerization by severing, an activity that is inhibited by PIP₂ and PIP. At intermediate calcium levels villin caps the barbed end of filaments thus preventing elongation, and will nucleate filament growth if added to monomers (473). In the absence of calcium, villin has no effect on polymerization, but villin cross links actin into bundles (121). As the calcium concentration increases, bundles are dispersed as the filaments are fragmented by villin.

Villin is thought to participate in the assembly and maintenance of the intestinal brush border cytoskeleton, and with fimbrin dictates the structure and organization of the microvilli actin bundle. Transfection of human villin genes and the synthesis of large amounts of villin in cells which do not normally produce it, and which lack brush borders, induces the growth of long microvilli on the cell surface and the reorganization of F actin, concomitant with the disappearance of stress fibres. (317,122). This suggests a key role for villin in the morphogenesis of microvilli. The headpiece domain, which binds F actin, is essential for villin's morphogenetic effect (123).

1 D LATERAL BINDING PROTEINS

This class of proteins binds to the side of actin filaments and can mediate filament interconnections, connect filaments with other proteins or regulate the interaction of other actin binding proteins with actin (351, 414, 459)

1 D i Hisactophilin

Hisactophilin is a 13 kDa histidine rich (31/118 amino acids) actin binding protein found in *D discoides*. Its unusual amino acid composition is thought to be the basis of the proteins pH dependent, actin filament binding. 90% of the histidine residues lie at the surface of the molecule, therefore a shift in pH creates a large number of positive charges or leaves the protein unchanged. The protein is likely to bind to actin in its polycation form. At pH 6.5, actin binding is saturated and at pH 7.5, binding is abolished. At pH 6/6.5, even in the absence of Mg^{2+} and K^+ , hisactophilin will induce actin polymerization. At pH 7, hisactophilin induced polymerization is retarded and incomplete. Hisactophilin may act *in vivo* to signal the slight changes in intracellular pH which result from chemotactic signals to changes in the actin cytoskeleton. Hisactophilin can bind along F actin filaments at a 1 : 1 molar ratio and induce the formation of large filament bundles and this activity may be explained by the uneven distribution of histidines in two opposing patches on the molecular surface (167).

1 D ii Calponin

Calponin is a basic, 34kDa actin binding protein that is abundant in smooth muscle and is also found in association with the actin cytoskeleton in some non-muscle cells, such as 3T3 fibroblasts and platelets (29, 422) Calponin also binds tropomyosin and calcium/calmodulin (299). Four/five isoforms of varying pI have been identified in adult smooth muscle, appearing sequentially during smooth muscle differentiation, and a lower Mr isoform of calponin, α calponin (28 kDa) which is expressed in the smooth muscles of the urogenital tract is down regulated in benign smooth muscle derived tumors (leiomyoma) (98). Calponin is thought to act as a regulator of smooth muscle contraction by inhibiting actin activated myosin ATPase. This inhibition is enhanced by caldesmon, and reversed either by calcium/calmodulin binding or phosphorylation by protein kinase C or calcium calmodulin dependent kinase II which inhibit calponin:actin binding (465). Calponin may inhibit the interaction of actin with myosin by inducing a conformational change in the actin subunits, as binding of calponin induces a decrease in fluorescence of pyrene labelled actin

filaments (322). A calponin specific phosphatase has been isolated from smooth muscle (482). Calponin is a substrate for calpain I (443)
Interestingly, calponin shows some homology with the GAP binding site of Ras p21.(421)

1 D iii Troponin

Troponin is a complex of three subunits, troponin C (17.8 kDa), I (20.86 kDa) and T (30.5 kDa) which, with tropomyosin, constitutes the calcium dependent regulatory machinery associated with actin thin filaments in skeletal muscle. The binding of calcium to troponin C (via EF hands) induces a conformational change in the molecule which is transmitted to other thin filament components and results in the activation of actomyosin ATPase and *in vivo* force development and the contraction of muscle fibres. Troponin I inhibitory subunit inhibits actomyosin ATPase and moves away from actin when calcium binds troponin C. Troponin T tropomyosin binding subunit binds tropomyosin and thereby attaches the troponin complex to thin filaments. Troponins have not been identified in non-muscle cells. (160, 236)

1 D iv Tropomyosin

Tropomyosin is found in almost all eukaryotic cells in association with actin filaments (256). It exists as a dimer of identical or similar α helical polypeptides arranged in parallel as a coiled coil and appears as a rod-like molecule and it lies along side the two long pitch helices of F actin (256). In skeletal and smooth muscle (the long isoform) tropomyosin spans seven actin monomers, while in non-muscle cells a short isoform spans six actin molecules (375). Both forms can be present in non-muscle cells. A pattern of six or seven, fourteen or twelve amino acids repeats in tropomyosin corresponds to six or seven actin subunit binding sites allowing tropomyosin to bind along the filament (256). Tropomyosin molecules polymerize head to tail and bind cooperatively to F actin. (175) In skeletal and cardiac muscle, tropomyosin, together with the troponin complex, is a component of the calcium sensitive regulatory mechanism controlling actin : myosin interactions and hence muscle contraction (256, 175). Tropomyosin in skeletal muscle stabilizes thin filaments by slowing depolymerization from the pointed end filament and

may enhance the stability of thin filaments (479). Its role in smooth muscle and non-muscle cells where the troponin complex is absent is less clear, but its interaction with caldesmon, calponin and tropomodulin suggests an important regulatory role in these tissues (465).

Tropomyosin in non-muscle cells can stabilize actin filaments, inhibiting their spontaneous fragmentation and increasing their rigidity (351). Tropomyosin blocks the interaction of cross linking proteins, such as filamin and α actinin and a 55 kDa bundling protein. In addition, tropomyosin coated filaments are resistant to severing by gelsolin or villin and disassembly by actin depolymerising proteins such as ADF (261). Tropomyosin is absent from actin filaments at cell junctions, attachment plaques and the foci of polygonal networks in spreading cells, sites where α actinin is associated with actin (249). Tropomyosin competes for actin binding with villin and is found in terminal web of brush borders, where actin filaments are not packed into bundles by villin (119). In erythrocytes, the short actin filaments of the membrane cytoskeleton are supported by tropomyosin (6). Tropomyosin binds to only one filament and therefore does not act as a cross-linking or bundling protein (351).

A role for tropomyosin in the assembly and organization of actin filament bundles has been suggested by the lack of stress fibres in transformed kidney cells, which do not express tropomyosin, and by the lack of actin cables in yeast tropomyosin null mutants (261). Tropomyosin exhibits extensive cell type specific isoform diversity with distinct isoforms found in muscle, brain and non-muscle cells (115,256). This tissue specific expression suggests that each isoform is required to perform specific functions in various muscle and non-muscle cells. Three major and two minor isoforms are found in fibroblasts, where low Mr isoforms which have a low affinity for actin and are found in membrane ruffles and stress fibres, while high Mr isoforms which have a high affinity are found only in stress fibres where they display a greater protective effect against, for example, gelsolin severing (261,492). High Mr tropomyosins can dissociate gelsolin caps from actin and allow annealing of gelsolin severed/capped fragments in the presence of calcium (204). This effect is potentiated by non-muscle caldesmon, which also enhances tropomyosin binding and enables low Mr tropomyosins to reverse gelsolins severing action (492). A correlation

between morphological changes and changes in expression of tropomyosin is seen in oncogenically transformed cells (262,492) where the down regulation of high Mr tropomyosin correlates with the loss of stress fibres. Changes in non-muscle actin gene expression can effect the cytoskeleton organization and expression of tropomyosin in an isoform specific manner (386).

1 D v Caldesmon

Caldesmon is a multi-functional, extended dumbbell shaped protein found in smooth muscle (87 kDa) and many non-muscle cells (65 kDa) where it binds specifically to actin, calmodulin, tropomyosin and myosin and is also a substrate for many protein kinases (284). Smooth muscle and non-muscle caldesmon are incorporated into the stress fibres, microspikes and membrane ruffles of non-muscle cells, but skeletal muscle cells actively exclude caldesmon from thin filaments (490). Caldesmon is thought to be involved in the regulation of smooth muscle contraction, as it inhibits actomyosin ATPase activity by binding actin filaments and blocking myosin interaction via competition for the N terminus of actin with myosin (460,63,293). This inhibition is enhanced by tropomyosin and lost upon phosphorylation of caldesmon by calcium/calmodulin dependent kinases or binding of calcium/calmodulin to caldesmon, which dissociates caldesmon from actin (47,170). Because caldesmon can bind to actin and myosin, it has been suggested that it cross-links thick and thin filaments and allows the maintenance of steady tension in some muscles via a latch mechanism (132,284). The affinity, but not stoichiometry (1:6 - 1:12 caldesmon : actin) of actin binding by caldesmon is enhanced by tropomyosin (133). Non-muscle caldesmon is found in membrane ruffles and stress fibres where it has a periodic distribution on stress fibres coincident with tropomyosin and complementary to α actinin. It binds alongside tropomyosin and is potentially in contact with actin:tropomyosin along its entire length (284). Caldesmon stimulates the binding of low Mr tropomyosin isoforms to actin and, in combination with tropomyosin, reverses the severing/capping effect of gelsolin. This, and the cross-linking of actin to myosin by caldesmon (492), increases the stability of the stress fibres. Caldesmon is able to associate into dimers /oligomers via disulphide bonds and acts as an actin bundling protein which also increases the stability of

the actin filament bundles (329,46,293,). This activity is reversed by calcium/calmodulin . In cultured cells, extensive changes in microfilament organization are seen at mitosis, with the disassembly of stress fibres and the transient formation of a contractile ring. Caldesmon is released from stress fibres during mitosis following mitosis specific phosphorylation of caldesmon by p34 *cdc2* kinase (445,494). Caldesmon does not localize with the contractile ring, but becomes diffusely distributed through the cytoplasm and as it becomes dephosphorylated, reassociates with the microfilaments that form as daughter cells start to spread. This suggests a role for caldesmon in assembling and maintaining the integrity of interphase stress fibres. The release of caldesmon from stress fibres may allow actin : myosin interactions within the contractile ring to cleave the cell, and also relax the inhibition of gelsolins severing activity, which would allow actin filament reorganization. *In vitro*, caldesmon can promote actin polymerization and the assembly of myosin into filaments (369, 132).

1 D vi Cortactin

Cortactin (p80 p85) is an F actin binding protein that is enriched in the cell cortex, especially protrusive structures, such as ruffles and lamellipodia, of many adherent cells and it co-distributes with F actin into the invadopodia of transformed cells. It is phosphorylated to high stoichiometry (30%) in RSV transformed cells and cells stimulated with growth factors, and while tyrosine phosphorylation does not inhibit its actin binding it may stimulate the binding of cortactin to proteins containing SH₂ domains in, for example, the invadopodia of transformed cells and thus induce aberrant clustering of actin in these regions. Cortactin may have a role in regulating microfilament : membrane interactions in response to cell surface signals propagated by receptor and non-receptor kinases. An SH₃ domain, a potential membrane localization site, may link cortactin to membrane protein (489).

1 D vii Adducin : (PK1, PK2, 115/110, Cam BP)

Adducin is a dimeric molecule composed of α (103kDa) and β (97 kDa) subunits which is found in a wide variety of cells and tissues. It is associated with the membrane skeleton of

erythrocytes. It binds to the sides of actin filaments and at high concentrations will bundle actin filaments. It has been suggested that adducin plays a role in the assembly of spectrin actin networks by promoting the binding of spectrin and actin. It binds to spectrin : actin complexes with a higher affinity than it binds either protein alone and the resulting ternary complex promotes the binding of a second spectrin molecule to the complex. This spectrin recruitment is inhibited by calcium-calmodulin, suggesting a role for adducin in mediating calcium-calmodulin dependent reorganization of the cytoskeleton.

Adducin is found at the lateral cell borders of epithelial cells, including MDCK cells and keratinocytes and human colon carcinoma cells. It is one of the first proteins recruited to sites of calcium dependent cell : cell contact in keratinocytes, suggesting that it may have a role in organization of cell junctions, and is also present in leukocytes where it may have a role in the local reorganization of the membrane skeleton at sites of phagocytosis. (236)

1 D viii Tenuin

Tenuin is a high Mr (400 kDa) protein localized at the cytoplasmic face of cell : cell adherens junctions and is concentrated in actin bundles, such as stress fibres and circumferential bundles in epithelial cells. It is suggested that it has a key role in forming and maintaining the structural integrity of actin filament bundles in non-muscle cells.(236, 269)

1 D ix Nebulin

Nebulin is a giant (800 kDa) skeletal muscle specific, actin binding phosphoprotein that functions as a length regulating template for the actin thin filaments. Nebulin attaches to the Z line of the sarcomere via its C terminus and binds with high affinity to actin filaments to form a composite thin filament. The protein does not extend and also has binding sites for α actinin and calmodulin. (436)

1 E CROSS-LINKING PROTEINS

Some but not all of these proteins can cross-link actin filaments into isotropic networks, the loose open structures of orthogonally oriented filaments that can resist deformation and

confer gel like viscoelastic properties to the cytoplasm (476, 170). Other actin cross linking proteins bundle actin into parallel arrays of more closely packed filaments (170). For some proteins, for example, α actinin (75) this is a function of concentration, and at low molar ratios to actin, it induces network formation, and at higher concentrations parallel alignment of filaments occur to form bundles (476). In most instances, however, the type of actin cross-linking produced is a function of size, and the arrangement of actin binding domains. An actin cross linking protein must have two actin binding sites, with each binding to a separate actin filament. The differential alignment and spacing of actin binding domains allow proteins to create different filament ultrastructures. In general, smaller globular or short, rod-like proteins (fascin, fimbrin, villin) where actin binding domains are not well spaced, will pack filaments into bundles *in vitro*, and are associated with actin bundles in microvilli and filopodia, (476) while large flexible proteins, for example filamin, can promote a high degree of branching between filaments *in vitro*. because the actin binding domains are separated, often by a flexible rod region. Such large proteins occur predominantly in isotopic gels (82, 476), and reside at the vertices of branches in orthogonal cortical actin networks (417).

Many actin cross-linking proteins share a homologous actin binding domain and belong to a common family which includes α actinin, spectrin, fimbrin, filamin, ABP 120 and dystrophin. Differential arrangement of the actin binding domains, spacer or rod domains which consist of variable numbers of an α helical (rigid) or β sheet (flexible) motif and extend the distance between actin binding domains and provide a region for interaction with other proteins, oligomerization domains, and calcium and membrane binding domains produce actin cross-linking proteins with different properties . Thus the large differences in actin assembly made by these proteins with similar actin binding domains emphasise the importance of how these domains are arranged within the molecule (292, 170, 100).

1 E i Small Actin Binding Proteins, Gelactins, ABP30

These are polypeptides of Mr 35 kDa or less, that cross-link actin filaments into either isotopic networks or bundles, and are found in *Dictyostelium* and *Acanthamoeba* and higher eukaryotes.

ABP 30 from *D. discoides* is a calcium sensitive, 34 kDa monomeric actin bundling protein. Direct binding of calcium via two EF hands motifs induces a decrease in the affinity of actin binding by ABP 30 and therefore inhibits the formation of cross-linked actin structures. In addition, this molecule appears to inhibit F actin depolymerization at both the barbed and pointed ends of the filament. The ends appear to be free for assembly but not disassembly and this inhibition of depolymerization does not appear to depend on cross-linking and is not regulated by calcium.

The localization of ABP 30 to filopodia and anterior pseudopodia of amoeba, around the phagocytic cup and regions of cell to cell contact in developing cells suggest that it has a role in organizing the structure of F actin and may contribute to the regulation of dynamic behaviour of the cortical cytoskeleton by promoting the formation and net accumulation of filament arrays with enhanced stability in specific locations (502).

An antigenic homologue with Mr 34 kDa has been found in mouse 3T3 and rat kidney fibroblasts which it is localized at the cell surface and in stress fibres in these cells and it is thought to act as an actin bundling protein.

A number of other small actin cross-linking proteins have been described. Four actin cross-linking proteins, the gelactins, with Mr of 23, 28, 32 and 38 kDa have been isolated from *A castellanii*. Each one can gel actin at low levels (289).

A 30 kDa actin bundling protein, p30b, found in *D discoides* is distinct from ABP 30.

A 36 kDa protein from *Physarum* binds to actin at a molar ratio of 1:7 actin monomers, cross-links actin monomers into the tangled aggregates seen at the onset of the relaxation phase of cytoplasmic streaming and inhibits the interaction of actin and myosin. It may therefore have a role in regulating force generation (328).

1 E ii Dematin (Band 4.9)

Dematin is a trimeric phosphoprotein of 3 x 48 - 52 kDa subunits. It is a stable component of the mature erythrocyte membrane skeleton, one trimer binding to the side of the actin oligomer, and it may participate in cytoskeleton reorganization during maturation. It is also present in many vertebrate tissues, where it is thought to link the membrane skeleton to the plasma membrane via its association with a membrane protein. *In vitro*, purified dematin

binds to and bundles actin filaments, an effect which is reversibly inhibited by phosphorylation. Although dematin does not bind G actin *in vitro*, it increases the lag phase of polymerization and decreases the rate of elongation. (192)

1 E iii Fascin

Fascin is a 58 kDa monomeric (P&C) calcium insensitive, actin filament cross-linking protein that is found to bundle actin in sea urchin egg microvilli, coelomocyte filopodia starfish oocytes, *Limulus* sperm acrosomal processes and pig brain . *In vitro*, it binds to the side of actin filaments to form needle-like structures comprised of parallel arrays of filaments cross-bridged at regular intervals and is presumably responsible for the organization of filament bundles *in vivo* (351, 292).

1 E iv MARCKS (Myristylated alamine rich C kinase substrate)

MARCKS is an acidic, rod shaped phosphoprotein of 68 - 87 kDa whose actin cross-linking activity is regulated by phosphorylation and calcium calmodulin binding (171). MARCKS is associated with sides of actin filaments at their points of contact with the plasma membrane, in for example focal contacts. Although both phosphorylated and dephosphorylated MARCKS bind to actin, dephosphorylated MARCKS has a greater affinity for actin and is able to cross-link actin filaments into bundles. When phosphorylated, MARCKS redistributes from the plasma membrane to the cell interior, and on dephosphorylation reassociates with the plasma membrane, where it is able to aggregate and cross-link actin filaments to maintain attachment to the membrane. An additional level of regulation is brought about by calcium/calmodulin binding to the dephosphorylated MARCKS, which inhibits its actin cross-linking activity and prevents protein kinase C phosphorylation (171, 215,216)

MARCKS is specifically phosphorylated during neurosecretion, growth factor dependent mitogenesis (eg with PDGF) or agonist induced cell activation of macrophages or neutrophils in parallel with protein kinase C activation and increased calcium levels.(171, 215). Interestingly, MARCKS expression is down regulated in v-src transformed cells and this suggests that the physiological function of MARCKS is maintaining flat

nontransformed phenotype, and modifying actin : plasma membrane interactions in response to various stimuli (171, 215,4).

1 E v HSP 90 and HSP 100

In vitro, these proteins co-precipitate with skeletal muscle F actin, increase its low shear viscosity and are therefore assumed to cross-link with actin. HSP 90 is localized in membrane ruffles and the further inside the cytoplasm, but is readily displaced from stress fibres by tropomyosin. It is a carrier for pp60 src and a steroid hormone receptor binding protein. This suggests that actin binding may be important in the intracellular transport of various functional proteins and that actin binding proteins may have additional roles (233).

1 E vi Lymphocyte Specific Protein -1 (LSP-1)

A lymphocyte specific phosphoprotein, LSP1 is a 52 kDa calcium binding protein that is associated with the cytoplasmic face of the plasma membrane and the cytoskeleton via its direct binding to the side of actin filaments .

In vitro, it does not bind to G actin, nor alter polymerization kinetics of actin, but it does induce F actin bundling. The basic C terminal domain required for actin binding is similar to the F actin binding fragment of caldesmon. Its localization beneath caps and its association with both the membrane and the cytoskeleton suggest that it may have a role in mediating the cytoskeleton remodelling that occurs after B cell activation, receptor capping, cell motility and cell : cell interactions (214).

1 E vii Fimbrin (Acumentin / L & T Plastin / SAC 6 CY/yeast ABP 67)

Fimbrin is a globular, monomeric, 68 kDa protein that was originally identified as a major component of the actin bundle in brush border microvilli of intestinal epithelial cells (142). It is also enriched in actin cytoskeleton-containing structures, such as microspikes and filopodia, stereocilia of hair cells, membrane ruffles and cell : cell and cell : substrate adherence sites of essentially all non-muscle cells (292, 142). *In vitro*, purified fimbrin cross-links actin into tightly packed, relatively straight, rigid bundles (42). The bundling of actin filaments by villin and fimbrin results in polar structures. This observation, and

the expression of fimbrin during development and intestinal cell differentiation, strongly suggests an *in vivo* role for fimbrin in the formation and organization of polar actin filament bundles. Fimbrin actin binding is influenced by pH, buffer composition, ionic strength, concentration of magnesium and calcium, and phosphorylation (142). For example, binding is inhibited by 100mM KCl and calcium, although as the calcium site can be saturated by magnesium, fimbrin is less sensitive to calcium than villin. (42,142). Fimbrin phosphorylation *in vivo* may have a role in the regulation of the membrane association of the cytoskeleton . L-plastin (the human homologue of fimbrin) is phosphorylated on leukocyte activation (92). Sequence analysis has revealed that fimbrin is a member of the α actinin family of cross-linking proteins which have homologous actin binding domains . Fimbrin has N-terminal two calcium binding EF-hand motifs, no rod domain and a tandem arrangement of two actin binding sites at C-terminus that leads to the close packing of actin filaments in bundles.(459,100)

1 E viii Actin Binding Protein 120 (ABP 120) (Gelation factor)

ABP 120 is an abundant protein from *D. discoidea* that *in vitro*, cross-links actin filaments into an orthogonal network in a calcium-insensitive manner. Its localization to the cell cortex, specifically in newly formed pseudopodia following cAMP stimulation (323), and the poor motility and pseudopod extension of mutant cells lacking ABP 120 suggests that the *in vivo* function of ABP 120 is the formation of filament networks during cell locomotion. ABP 120 is a member of the α actinin family of cross-linking proteins (100, 292) . It exists as a rod-like homo-dimer of 2 X 120 kDa antiparallel subunits (323). Each subunit has an actin binding domain site at the N-terminus, and a rod domain of six β pleated sheet motifs, which is more flexible than the rod domain of α actinin, enabling ABP 120 to form actin networks rather than bundles of actin filaments. Dimerization is via hydrophobic side chains (323, 170) .

1 E ix α Actinin (Actinogelin)

α actinin is an F-actin bundling protein originally isolated from skeletal muscle where it is a component of the Z-disc (30). It is found in muscle and non-muscle cells at points where

the actin is anchored to a variety of intracellular structures, so that in smooth muscle α actinin is found in membrane associated dense plaques and cytoplasmic dense bodies, in cardiac muscle in fascia adherens of intercalated discs and Z discs and in non-muscle at adherens type cell : cell and cell : substrate junctions where it is part of the cascade of molecules that link the actin cytoskeleton to the plasma membrane. In addition, it has a periodic distribution alternating with tropomyosin along the stress fibres in the fibroblasts and in the terminal web of intestinal epithelial cells (30). F actin bundling by α actinin in contractile stress fibres allows actin : myosin interactions (292). α actinin exists as a rod shaped homodimer of 2 x 94 - 103 kDa subunits arranged in anti parallel orientation so that the N terminal actin binding domains lie at opposite ends of the molecule (30). The rod or spacer domain is comprised of four α helical motifs and is the site of oligomerization and interaction with vinculin, nebulin and the cytoplasmic domain of β 1 integrin subunits (292). Two calcium binding EF hand motifs lie at the C terminal of each subunit. Only the actin binding of the non-muscle isoform is calcium sensitive, and muscle isoforms are not regulated in this manner (30). Tropomyosin can inhibit α actinin binding to actin. α actinin isolated from the Z lines of striated muscle is found to contain endogenous PIP_2 and the addition of PIP_2 to smooth muscle α actinin can enhance its activity to that of skeletal muscle isoforms (125).

1 E x Spectrin (Fodrin, TW 260/240)

Spectrins are a family of actin binding proteins, mainly found in association with the plasma membrane of mature cells. In addition to binding to itself and actin, spectrin also binds ankyrin, band 4.1, adducin, glycoproteins and calcium calmodulin. Spectrins bestow mechanical stability to the plasma membrane and link membrane proteins (and possibly phospholipids) to the cortical actin cytoskeleton. Spectrin exists as a rod shaped heterodimer comprised of antiparallel subunits (α and β) which form tetramers ($\alpha \beta$)₂ by end : end association of the N terminus of α chains and the C terminus of the β chains. The actin binding domains that lie at the N terminal of β chains are missing from α chains, so that formation of a tetramer is essential for actin cross-linking. Two EF hands lie at the C terminus of the α chain. The rod domain consists of multiple repeats of an α helical

motif (292, 151, 100). There are three functional classes of spectrin. Erythrocyte spectrin was first isolated from human red blood cells where spectrin tetramers are bound together by short actin filaments and attached to glycoproteins (band 3) by ankyrin and band 4.1, to form a two dimensional lattice at the cytoplasmic face of the red blood cell membrane. TW 260/240 is found in the terminal web of avian intestinal brush borders, where they cross-link the actin bundles into a network, but do not associate closely with the plasma membrane (20, 74). Non-erythroid spectrin, known as fodrin, is found in the cortical cytoplasm of many cells where it may be confined to distinct membrane domains and is thought to organize receptor domains (as it co caps with surface antigens in lymphoid cells) and to control vesicle trafficking at the plasma membrane. It links the actin cytoskeleton to the plasma membrane via its association with transmembrane proteins (143, 74). *In vitro*, the F actin binding and cross-linking of fodrin is influenced by ionic strength. The fodrin : actin interaction is also regulated by calcium and calmodulin binding, phosphorylation and calpain I proteolysis following cell stimulation for example in activated platelets and degranulating neutrophils, and it has been shown to transfer between the cytoplasm and membrane in response to external stimuli (20, 308).

1E xi Actin Binding Protein 280 (ABP 280) / Filamin

ABP 280 is an elongated, V shaped, homodimeric phosphoprotein of 2x 280 kDa subunits which cross-links and promotes perpendicular branching of actin filaments to form orthogonal networks in the cortical cytoplasm (466,467) . It also tethers the actin network to the membrane via binding at its C terminus to membrane glycoproteins (170,476), for example, in platelets, to the Gp1b/IX complex (467). The dimerization sites are at the extreme C-terminus of each subunit, and the N-terminal actin binding domains are unconstrained and are separated by the very flexible spacer/rod domain, which consist of 23 tandem repeats of β pleated sheet motif (170) which have hinge-like regions (161). Therefore actin filament branching at high angles and the formation of isotropic gels is enabled by ABP 280 crosslinking. (476, 170, 292) Neither calcium nor phosphorylation state appear to regulate filament binding, although the extent of ABP 280 phosphorylation

changes in response to cell activation. cAMP dependent protein kinase activity appears to protect the molecule against calpain proteolysis (161).

ABP 280 has a role in the stabilization of the cortical cytoskeleton and membrane, the maintenance of cell shape and the regulation of cell motility. Melanoma cells in which ABP 280 expression is down regulated show uncontrolled membrane blebbing and migrate poorly in response to external stimuli. ABP 280 binds directly to the Gp1b/IX complex and mediates cell attachment to external glycoproteins, such as von Willebrand factor which is immobilized on damaged vascular walls, and therefore has a role in tissue repair. The binding interaction between the Fc γ R1 high affinity IgG1/ IgG3 receptor and ABP 280 is inhibited by receptor/IgG binding. In this way the signal transduction pathways for antibody mediated functions of phagocytes and lymphocytes can be linked to the actin cytoskeleton (329).

1 E xii Dystrophin

Dystrophin is a large (427 kDa) member of the α actinin family, and is associated with the plasma membrane in muscle and neuronal tissue. It exists as a rod shaped antiparallel dimer. Each subunit of the skeletal muscle isoforms has an N terminus α actinin-like actin binding domain, a large rod domain of 24 α helical repeats that promote dimerization and may be elastic, a cysteine rich region with two non-functional EF hands and a fourth distinct region that may mediate plasma membrane attachment of dystrophin via a complex of integral membrane glycoproteins (292, 170). Fatty acid acylation of the cysteine rich residues may contribute to the strength of dystrophins plasma membrane attachment (50, 105, 104, 181).

Dystrophin is localized to the cytoplasmic face of the plasma membrane, including neuromuscular and myotendinous junctions in muscle cells; it binds peripheral actin filaments emanating from Z and M lines of skeletal muscle myotubes and links them to the plasma membrane and actin filaments to laminin via the membrane glycoprotein complex (50). By analogy to spectrin, and because of the progressive pattern of damage to the muscle membrane in Duchennes muscular dystrophy, it was thought that the function of dystrophin in skeletal muscle might be to stabilize the membrane and provide resilience to

cycles of contraction and relaxation (104,269). It appears, however, that dystrophin and the associated actin cytoskeleton are part of the network designed to detect and respond to the tensions in the myofibrillar lattice that occurs as the bone skeleton enlarges and lengthens during normal post natal growth, rather than those generated by contraction and relaxation of the muscle. This tension is transmitted to the myotendinous junction where it induces sarcomeres to be added to the ends of striated muscle myofibrils. The inability of dystrophin deficient muscle to keep pace with the enlarging skeleton, especially during periods of fast growth in adolescence, seems to be responsible for the progressive degeneration of skeletal muscle in Duchennes muscular dystrophy (50). In brain, the restricted localization of specific dystrophin argues for a function in synaptic organization. Dystrophin isoforms with alternatively spliced C terminus and lacking N or rod domains are expressed at high level in non-muscle tissue and their function in these is unclear (104).

1 F MEMBRANE ASSOCIATED ACTIN BINDING PROTEINS

1F i Ponticulin

Ponticulin (1x 17 kDa) is a glycoprotein found throughout the plasma membrane and in intracellular vesicles in *D. discoides* amoebae and in human leukocytes (67,270). It forms a major link between the cytoskeleton and the plasma membrane in *D. discoides* amoebae by binding directly to the sides of the F actin (67,344). *In vitro*, ponticulin : actin binding is diminished in high salt suggesting the interaction, at least in part, is electrostatic in nature, and it can also be inhibited by reducing agents, suggesting that disulphide bonds are also important (67). By clustering into oligomeric complexes in the plasma membrane, ponticulin is thought to nucleate the assembly of actin filaments, which have both ends free, at the cytoplasmic faces of the plasma membrane (67,395). Ponticulin is enriched at the plasma membrane of nascent pseudopodia and regions of cell : cell contact (647, 280), and the amount of ponticulin present in the plasma membrane increases two to three fold during aggregation streaming of *D. discoides* amoebae, suggesting that ponticulin has a role in cell : cell adhesion and/or cell motility. Nucleation of actin by ponticulin may be regulated by the activity of the second messenger, DAG (269,270,435).

1 F ii Actolinkin

Actolinkin (1x 20 kDa) is found in the cortical region of sea urchin eggs and starfish oocytes. It seems to anchor actin filaments at the cytoplasmic face of the plasma membrane at their barbed ends. The ability of the sea urchin egg cortex to induce actin polymerization is lost after extraction of actolinkin and this addition of actolinkin or an actolinkin : actin complex restores polymerization activity. In the egg, the majority of actolinkin is thought to exist in a 1 : 1 complex with Gactin, which, *in vitro*, can bind to and block monomer addition and reannealing at the barbed end of the actin filaments. (236).

1 F iii Synapsin I

Synapsin I is an 80 - 84 kDa protein that binds to synaptic vesicles that contain acidic phospholipids. It can also bind laterally to and bundle F actin (455). Its interaction with fodrin in brain suggests that it may link the actin cytoskeleton to the plasma membrane in a manner analogous to ankyrin. Synapsin I actin binding and bundling activity is inhibited by phosphorylation . It binds microtubules and neurofilaments and calmodulin and probably regulates neurotransmitter release from nerve endings. (455)

1 F iv Annexins

Annexins (calpactin, lipocortin, calcimedins, calelectrins and synexin) are a family of 35 kDa proteins that bind acidic phospholipids in a calcium sensitive manner. They also bind actin and spectrin, suggesting that they may have a role in the connection of cortical actin cytoskeleton to the plasma membrane.(236)

1 G OTHER ACTIN BINDING PROTEINS

1 G i Protein 4.1/ Band 4.1

Protein 4.1 (78 kDa) was originally isolated from red blood cells where it promotes the association between spectrin and actin in a calcium/calmodulin dependent manner. It links the spectrin lattice to the membrane of the red blood cell via its interaction with glycophorin and therefore has an important role in maintaining membrane stability and cell shape. Non-erythroid 4.1 isoforms are likely to have similar roles in attaching the cytoskeleton to the

plasma membrane, and to the mitotic apparatus as protein 4.1 isoforms appear to be associated with centrosome. Phosphorylation of band 4.1 by protein kinase C inhibits binding of band 4.1 with the cytoplasmic domain of the anion channel (270) and phosphorylation status also regulates the ability of band 4.1 to stimulate the spectrin : actin association. Band 4.1 is also regulated by phosphatidylinositol binding .

1 G ii Ezrin (Cytovillin, p81)

Ezrin (80 kDa) is a membrane associated protein that is enriched in actin-containing surface structures, such as microvilli of the intestinal brush border, membrane ruffles and the microspikes of the growth cone. It is a member of the moesin/radixin/band 4.1 family, which suggests that it may link actin filaments to the plasma membrane (45). Ezrin is a substrate for the tyrosine kinase activity of the EGF receptor and other kinases, and a correlation exists between the phosphorylation of ezrin by EGF receptor kinases or DAG activated protein kinase C and the appearance of cell surface structures, to which phosphorylated ezrin is rapidly recruited. (45,437, 270)

1 G iii Moesin (membrane organizing extension spike protein)

Moesin is a 78 kDa member of the 4.1/talin/ezrin family, and is thought to have a similar role as a structural link between the plasma membrane and the cytoskeleton. It has a diffuse distribution on or near the cell membrane and is preferentially localized to cell surface protrusions such as blebs, microspikes, filopodia and lamellipodia (311, 437).

1 G iv Merlin/Schwannomin

Merlin/schwannomin shows striking similarities to members of the moesin, ezrin, radixin family and therefore it has been suggested that it links the cytoskeleton to the plasma membrane. Its expression is down regulated in neurofibromatosis 2, which is characterized by development of central nervous system tumours (437, 370).

1 G v Nuclear Actin Binding Protein (NABP)

NABP is a nuclear protein composed of 2 x 34 kDa subunits that has a diffuse nucleoplasmic staining pattern with some concentrated around, but not within, the nucleolus of human skeletal muscle, glial cells, retinal epithelial cells, *Acanthamoebae* and xenopus oocyte nuclei. It binds both actin and DNA. Xenopus NABP reduces the viscosity of actin gels in a calcium insensitive manner, but its physiological role is unknown. (8,367)

Other nuclear actin binding proteins include myosin, cofilin, Cap Z, g cap 39 and HSP 90/100 (333).

1 G vi Coronin

Coronin is a 55 kDa actin binding protein from *D. discoides* that binds to F actin in a calcium dependent manner. It is concentrated in the crown shaped projections of the actin rich cell cortex on the dorsal cell surface and is enriched at the site of pseudopodia extension in cAMP stimulated aggregating cells. It shows similarities to the β subunits of the heterotrimeric G proteins and may provide a link between the G protein complex of cAMP receptor and the cytoskeleton (94).

1 H MICROTUBULE BINDING PROTEINS

1 H i Tau

Tau is a microtubule associated protein which, when dephosphorylated, promotes the assembly of microtubules from tubulin subunits. It is found specifically in neuronal cells along the length of the microtubules, and regulates microtubule dynamics during neurite outgrowth, but can also bind to actin filaments. Tau is a major component of the neurofibrillary tangles which are a common feature of Alzheimers disease. (295)

1 H ii MAP 2

MAP 2 is found specifically in neuronal cells and it binds microtubules and promotes their assembly from tubulin subunits . It has actin bundling activity and is associated with actin in dendritic spines which lack microtubules.(295)

1 I MOTOR PROTEINS

Motor proteins hydrolyse nucleotides and harness the derived energy to move (unidirectionally) along cytoskeletal polymers. Myosins are a superfamily of related motors that move along actin tracks. The ability to recognise the asymmetry of actin filaments (movement is towards barbed ends) allows myosin in the cell cortex to transport membrane glycoproteins towards the leading edge of migrating cells, direct and transport membrane bound vesicles or, if anchored, to generate tension in the opposite direction from the membrane. Cells contain a range of myosins which are used for actin based movements (434). All myosins are characterized by a conserved 80 kDa head domain that can bind actin, hydrolyze ATP and translocate along actin filaments, and a neck domain of a variable number of tandem repeats, each of which may be light chain binding domain. These define functional specificity of a given myosin binding site and/or define the native oligomeric state of a given myosin.(305)

Myosins have a variety of structurally distinct, functionally specialized C terminal tail domains (434,65) that are responsible for localization of various myosins within the cell and may also specify interactions with transported component.

Traditionally, myosins have been classified into two groups, depending on their ability to form dimers/filaments. Myosins II are the conventional, two headed, filament forming myosins of muscle, while myosin I are smaller monomeric myosins, first discovered in *Acanthamoeba*. Genetic analysis, however, has suggested that the currently known myosins should be divided into at least eight major classes, myosins I - VIII, based on their head domain sequences and the tail domain structures (305,434, 15).

1 I i Myosin I

Myosins I form a group of relatively small, monomeric and therefore single headed, non-filament forming myosins, the first example of which was discovered in *Acanthamoeba* (231). Mg ATPase activity is regulated by phosphorylation by myosin I heavy chain kinase (231, 434). The group is quite diverse, with at least three sub-divisions (65). The original *Acanthamoeba* myosin I was shown to have an actin binding S₁ head domain with actin activated Mg ATPase activity, tail domains that contain an ATP independent actin binding site and a membrane binding site. Molecular cloning of *Acanthamoeba* myosins

IB and I/C and Dictyostelium myosin IB has revealed that the tails of these myosins contain three domains; a highly basic membrane (and anionic phospholipid) binding domain, a GPA/GPQ rich domain (which may contribute to the actin binding site) and a SH₃ domain, which is present in a number of membrane cytoskeleton actin associated proteins) (231,65). These myosins are associated with both the plasma membrane and actin in lamellipodia and pseudopodia at the leading edge of migrating cells (126), phagocytic cups and intracellular membrane vacuoles. For example, *Acanthamoeba* myosin 1C is associated with the contractile vacuole (97, 231, 434,65). *In vitro*, these myosins will support the movement of latex beads or membrane vesicles along actin cables. These observations and experiments with null mutants suggest that they play an important role in phagocytosis, organelle movement and cellular translocation.

Another type of myosin I has been identified in *D. discoides*. This myosin has a short tail which contains a membrane binding domain but not the GPA/GPQ (phospholipid) and SH₃ domains and therefore probably lacks a second actin binding site, (65) but it can still link actin to membranes and may allow filament sliding (434). The first vertebrate myosin I to be identified was the brush border myosin I, whose expression is limited to tissues such as intestine that have brush borders. The tail is characterized by a highly basic neck region that contains 3 - 4 repeats of the calmodulin light chain binding domain. The rest of the (120 kDa) tail is very basic and contains a phospholipid membrane binding site, but not a second actin binding site (65,434). It is isolated with 3 - 4 calmodulin light chains that bind even in the absence of calcium. Calcium binding to calmodulin regulates and inhibits its MgATPase activity and slows motility. Brush border myosin I can bind actin and acidic phospholipids and it cross-links the actin filament bundle in the microvilli to the surrounding plasma membrane. This binding may be regulated by phosphoinositide signalling pathways and/or phosphorylation, as *in vitro*, when phosphorylated by protein kinase C it shows increased binding to phospholipid vesicles (434). This myosin does have mechanoenzyme activity (304, 434) and it has been suggested that it has a role in the delivery of newly synthesized membrane components to the apical domains of cells. Other vertebrate myosins I, similar to brush border myosin I, have been isolated from the cortex of bovine adrenal glands and mouse brain. In cells in culture (such as MDBK and CHO)

they are highly concentrated in motile extensions of the cell, such as lamellipodia and ruffles. Homologues have also been identified in *Drosophila* and yeast (MY03) (65).

1 I ii Myosin III

Nina C myosins are the most divergent of the myosin superfamily and so far have only been found in the photoreceptor cells of *Drosophila* eyes. They occur in two forms (174 kD and 132 kDa) by alternative splicing of the tail region. The larger form is localized to the microvillus-like rhabdomere where it may link actin filaments to the membrane and is thought to have a role in phototransduction, and the smaller isoform is found in the cell body. Nina C myosin has a unique 350 amino acid N terminal domain that is predicted to be a kinase whose role may be to regulate other rhabdomeric proteins important in phototransduction. The myosin domain would serve to traffic the kinase into the rhabdomere via its specific tail domain and maintain the rhabdomere structure. (354,65,15).

1 I iii Myosin IV

A high molecular weight myosin (177 kDa) isolated from *Acanthamoeba* is classified into its own group, based on the sequence of its head domain and its unique tail which lacks a membrane binding domain. Apart from an SH₃ domain at the C terminus, the tail shares no homology to the tail regions of other myosins. (65,305,15)

1 I iv Myosin V

This class of myosin includes mouse *dilute*, chicken myosin V (p190), yeast MYO4 and MYO2, which share a similar head domain and are characterized by a neck domain which contains 6 tandem repeats of putative calmodulin light chain binding sites. The tail domain has a proximal region which is predicted to form one or more segments of coiled coil α helix which may allow for some dimerization, and a globular distal domain. By immunofluorescence chicken myosin V appears to localize with intracellular membranes at the tips of growing glial and neuronal cells, and perinuclear regions of these cells near the Golgi apparatus. It is thought to traffic vesicles from the Golgi to the cell periphery and

deposit material at the plasma membrane. Yeast MYO2 null mutants are defective in budding, have disorganised actin and accumulate vesicles. In yeast, therefore, this myosin is presumed to be responsible for the polarized delivery of secretory vesicles to areas of active cell enlargement, such as the yeast bud. (65,305,15,434)

1 I v Myosin VI

The tail domain of *Drosophila* 95F and pig myosin VI, from a pig kidney proximal tubule cell line, contains a short a helical segment , which is predicted to form a coiled coil and may allow dimerization, followed by a unique region. *Drosophila* 95F has a punctate distribution in the cortex and cytoplasm of *Drosophila* embryo cells and is associated with unidentified organelles that redistribute during embryogenesis (305).

1 I vi Myosin VII

Myosins from *Drosophila* (35B,C) and a similar protein identified in a pig kidney cell line constitute this class. Only the head domain has been sequenced, and nothing is known of their tail structure or function (15)

1 I vii Myosin VIII

A myosin has recently been identified in flowering plant *Arabidopsis* (305).

1 I viii Myosin II

Myosin II molecules are isolated as hexameric molecules with two heavy chains and two pairs of light chains, the essential and regulatory light chains. They are characterised by the rod-like α helical tail of the heavy chain which allows them to form two headed dimers which can further assemble into bipolar antiparallel filaments. As with all myosins, the N terminal of the heavy chain is folded into a globular head with actin binding and actin activated MgATPase activity. Amoeboid (*Dictyostelium* and *Acanthamoeba*) myosin IIs are concentrated in the posterior half of locomoting cells (they can be visualised as thick filaments in the cortex of *D. discoides*) and in the cleavage furrow. They co-cap with ConA receptors. Disruption of the *D. discoides* myosin II gene results in cells that cannot divide

in suspension culture nor complete the development cycle. They are also defective in motile events. It is likely, therefore, that these myosins are involved in contraction of the cleavage ring during cytokinesis, establishing cell polarity, capping of cross linked surface receptors, and the generation of contractile tension in the cortex to drive cell locomotion.(342,65)

The remaining myosin IIs can be divided into two major groups, striated muscle myosin II and non muscle/smooth muscle myosin II. In non muscle cells of higher eukaryotes, the cytoplasmic myosins are likely to play a role in cytokinesis, receptor capping and cell locomotion. Myosin IIs in non muscle cells are found as filaments and as single molecules. Filament assembly is regulated by phosphorylation, so that when the regulatory light chain is dephosphorylated the myosin molecule assumes a folded, inactive conformation, but on phosphorylation by myosin light chain kinase it unfolds, can aggregate and has ATPase and motor activity. The tail region contains sites that direct spatial and temporal localization of the molecule in the cell. In striated muscle, myosin II aggregates to form the thick filaments of the muscle sarcomere and interacts with actin thin filaments to generate contractile forces. A number of skeletal muscle myosin II isoforms exist and within a single cell there are at least two isoforms of myosin light chain that can be differentially localized to the centre or the ends of the thick filaments. Different isoforms are expressed in fast and slow muscle, the speed of contraction is largely a function of the myosin heavy chain isoform, and in developing muscle. The function of myosin II can be regulated by phosphorylation or calcium binding. (65,398,438, 305)

2. ACTIN BINDING SITES

Distinct protein structures have been identified as actin binding domains and can be used to classify actin binding proteins into families. They are the myosin head, found in all members of the myosin family; the α actinin binding domain found in muscle and non-muscle α actinin, fimbrin (L-plastin), ABP120, ABP 280, spectrin, dystrophin and yeast fimbrin (SAC6p); the profilin domain; the gelsolin domain found in gelsolin and related proteins brevin, villin, gCAP39/ mbh-1, fragmin and severin; and the cofilin motif also found in ADF/destrin, chicken ADP, *Acanthamoeba* actophorin, and sea urchin depactin.

Other protein families, the tropomyosins, the caldesmons, the synapsins, and the Cap Z, cap 36/32 cap32/34, nuclear actin binding protein family all have unique actin binding domains. (459,236) Sequences of other actin binding proteins from different functional classes do not appear to be homologous with these actin binding units and there are a number of actin binding proteins which so far do not belong to a known class or cannot be grouped into a new class. With further study, it is likely that more classes of actin binding domains will be identified and it will be possible to group these actin binding proteins. (459,236)

The different actin binding domains are structurally unrelated when different classes are compared, suggesting that they bind to different sites on the actin molecule. However, actin subdomain 1 appears to be preferred target domain for many actin binding proteins, in particular the very acidic N terminus to which myosin, depactin, fragmin, cofilin, α actinin, troponin I, gelsolin, actolinkin and actobindin all bind. There appears to be no homology in these proteins at the sites that bind to the acidic N terminus except that they are rich in basic amino acids (459). The actin N terminus may function as a negatively charged area which attracts the charged clusters on actin binding proteins (459) and many basic proteins bind to actin, including aldolase and LSP1 and competition between various actin binding proteins for sites on actin could provide a mechanism for actin regulation (459).

Small actin binding motifs, found in actin binding proteins belonging to different classes, seem to exist. For instance, motifs similar to LKHAET sequences in actobindin, (which inhibits actin polymerization), are found in tropomyosin, thymosin β 4, fimbrin, α actinin and myosin light chain (453); the DAIKKK peptide, which inhibits the binding of cofilin to actin in a dose dependent manner is found in destrin, ADF actophorin and tropomyosin (497). LADYL is present in actin and several actin binding proteins. (459,236)

Common 'themes' in the amino acid sequence of actin, and some actin binding proteins that regulate actin assembly, suggest that actin binding proteins could inhibit the formation of filaments by 'molecular mimicry', directly binding to (or near to) a site on actin involved in actin : actin interactions. For example DESG in gelsolin is found at the C terminus of non-muscle actin, and similar tetrapeptides are found in fragmin, severin and muscle actin (DEAG), villin (DEQ) and also aldolase (DESG/T/V). The LTDYL sequence in actin which

may represent another actin : actin interaction site is also present in profilin, gelsolin, fragmin, severin, villin, vitamin D binding protein, cofilin and DNase I (75).

IV

1. REGULATION OF THE ACTIN CYTOSKELETON

Growth factors, chemoattractants, hormones and other extracellular molecules stimulate cells to reorganise their actin cytoskeleton, largely via the action of actin binding proteins which are in turn regulated by a variety of factors (206) such as phosphoinositide, second messengers and phosphorylation. (455).

1 A RECEPTOR STIMULATION OF POLYPHOSPHOINOSITIDE METABOLISM

Receptor stimulated phosphoinositide turnover, leading to the activation of protein kinase C, intracellular calcium transients and increases in phosphorylation are all involved in dramatic changes in cell shape and actin polymerization (117,401,41707 k8 Q. The production of at least two messengers (DAG and IP₃) by PIP₂ hydrolysis can be initiated by the stimulation of at least two different receptors (26). First, hormones or neuropeptides interact with and stimulate 7-pass membrane spanning receptors which are linked to heterodimeric GTP binding (G) proteins which dissociate into G α and G $\beta\gamma$ subunits. G α exchanges GDP for GTP and the activated GTP-G α binds and activates phospholipase C β 1, while the G $\beta\gamma$ subunit may activate another phospholipase, PLC β . Second, the growth factor receptor (EGF PDGF) has intrinsic tyrosine kinase activity, and growth factor binding induces dimerization of the receptor allowing two kinases to phosphorylate each other, creating a docking site to bind cytosolic proteins via its SH2 domains. The receptor kinase then phosphorylates and activates phospholipase C γ 1, which, by virtue of receptor binding, is close to and able to interact rapidly with its membrane bound PIP₂ substrate (27, 271).

One of the functions of activated phospholipase C isozymes is to hydrolyse PIP₂ to the second messengers IP₃ and DAG. IP₃ stimulates calcium transients, while DAG both activates protein kinase C and can induce nucleation of actin polymerisation at the

membrane. DAG and IP₃ are to a large extent recycled back to phosphatidyl inositol via two independent pathways and then by a two stage kinase phosphorylation via PIP, phosphatidyl inositol is converted back to PIP₂. By regulating PIP₂ hydrolysis by phospholipase C, profilin may play a role in phosphoinositide metabolism.(202,271,117). In resting cells, profilin binds cooperatively to a cluster of five PIP₂ molecules and protects them against the activity of unphosphorylated phospholipase C. IP₃ and DAG levels are therefore low. Following receptor stimulation phospholipase C is activated by phosphorylation, so that it can compete with profilin for PIP₂ interaction. Hydrolysis of some PIP₂ weakens profilin binding, which dissociates, exposing more PIP₂ to phospholipase C which leads to increased levels of IP₃ and DAG. On release from the membrane, profilin can interact with actin monomers and, by stimulating exchange of the nucleotide bound to actin, can increase the rate of actin filament assembly. Profilin may act in this way as an independent messenger to couple receptor activation to changes in the actin cytoskeleton, however, its participation in the signalling pathway *in vivo* is not yet established (271).

Growth factor receptors can also activate other effectors, namely a kinase which generates a putative lipid messenger PIP₃, and GAP which regulates ras activity (27).

1 B CONTROL OF ACTIN CYTOSKELETON BY POLYPHOSPHOINOSITIDE METABOLISM

Polyphosphoinositides, and the products of their metabolism, can interact directly with some actin binding proteins and control their activities. These interactions are important in both the assembly of actin structures and in phosphoinositide metabolism (170,455). The control of actin interactions by phosphoinositides was first described by Lassing and Lindberg. (244) Profilin : actin complexes interact specifically and with high affinity to membrane bound PIP₂ and PIP, whereupon polymerization competent actin is released from profilin at or near the membrane possibly due to a conformational change in profilin (455,117). The interactions of other actin monomer sequestering proteins, such as ADF/destrin, actophorin/depactin, (170) DNase I and cofilin (498) with actin are also inhibited by phosphoinositides, for example PIP₂ and PIP, which may decrease the affinity

of these proteins for actin, and inhibit their actin binding, by presenting a similar charged structure to that of the acidic N terminus of actin. Phosphoinositides can therefore affect actin polymerization by releasing monomers for assembly (170, 202).

Phosphoinositides, PIP and PIP₂ can also enhance actin assembly by binding with high affinity to actin filament capping proteins, such as Cap Z, Cap 100 (415), gCap39/MCP (271), fragmin, viilin and gelsolin, (202, 415) which reduces their affinity for actin, and therefore unblocks the fast growing barbed ends of actin filaments. PIP₂/PIP inhibit the calcium dependent severing activity of gelsolin (202,117) and, in addition, can dissociate gelsolin : actin complexes, even at low calcium levels and this interaction between calcium, PIP/PIP₂ and gelsolin allows the formation of different gelsolin : actin intermediates. Profilin and gelsolin compete for PIP₂ binding. (455,117).

Proteins that cross link actin are also shown to be regulated by phosphoinositides. For example, a actinin isolated from striated muscle is bound to PIP₂ which appears to regulate its actin crosslinking activity (125). Caldesmon interacts with acidic phospholipids in a calmodulin and phosphorylation dependent manner so that calcium/calmodulin inhibits the interaction of caldesmon with phosphatidylserine. Phosphatidyl serine displaces calmodulin from caldesmon and phosphorylation of caldesmon by protein kinase C decreases its affinity for phospholipids. Phospholipids inhibit the phosphorylation of MARCKS by protein kinase C so that MARCKS maintains its plasma membrane distribution and can continue to crosslink actin filaments and is at the plasma membrane(202).

By generating calcium signals, one of the products of PIP₂ hydrolysis, IP₃, acts as a second messenger to control many cellular processes (27). The other product of PIP₂ hydrolysis, DAG, which remains bound to the plasma membrane, can regulate actin assembly both by activation of protein kinase C and by indirectly enhancing actin assembly at the plasma membrane where it increases the rate of polymerization by mediating the formation of new nucleation sites, by interacting with one or more functional proteins. DAG may modulate the basal actin nucleation activity of ponticulin, or activate an unknown peripheral protein that directs the formation of nuclei (401,415) In addition, α actinin can interact with membranes containing DAG and palmitic acid and when inserted into a membrane, is protected against protease activity. (202).

When an agonist binds to a receptor, PIP₂ is hydrolysed, which might facilitate an association between profilin (and other monomer binding proteins) and actin. The generation of IP₃ releases calcium which activates gelsolin, which in turn severs or nucleates actin filaments, producing actin filaments with the barbed ends capped (and detaches them from the membrane). Phospholipase C binds to actin : gelsolin complexes and this interaction may target the enzyme to its substrate. This would promote filament disassembly and the severing of filaments to facilitate exocytosis of secretory granules and permit protrusive movement of the membrane (455,417). As levels of PIP and PIP₂ rise by phosphorylation of phosphatidylinositol and PIP, the barbed end capped F actin oligomers would diffuse to the plasma membrane and bind phosphoinositides via their capping protein. If calcium levels have fallen, PIP and PIP₂ would unblock oligomers by binding gelsolin. By binding profilin and other sequestering proteins that promote actin disassembly, PIP₂ releases actin monomers for elongation, (profilin catalyzes the nucleotide exchange on actin subunits) and together with cross-linking this would lead to the formation of an actin network (455,417,415).

Thus the accumulation of appropriate lipids at the leading edge or other sites may provide 'on' signal for actin assembly. Simultaneous uncapping of the barbed ends of the filaments and the release of monomers allow for rapid filament growth at the membrane sites where instructions for assembly originate and where assembly is required. Hydrolysis of phosphoinositides helps switch off assembly by increasing the availability of cappers and sequesterers (415).

1 C CONTROL OF ACTIN CYTOSKELETON BY CALCIUM

The intracellular level of calcium in resting cells is normally about 0.1 μM (117). A rise in this level, in response to cell stimulation, triggers many cellular processes with a high degree of spatial and temporal precision, including all cytoplasmic movements mediated by actomyosin interactions (90,179,392). Many actin associated regulatory proteins that control actin polymerization, network organization and actin filament membrane association are regulated by calcium (117) and they display calcium sensitive actin binding either by directly binding calcium or via the activity of a calcium binding protein (179). Changes in

the calcium level can affect the actin binding activities of a protein through calcium binding to an EF hand motif in the molecule, inducing a conformational change which is transmitted to and regulates the actin binding domain (90,455). For example, in non-muscle α actinin, the actin binding domain of one chain interacts with the EF hand of the opposite chain and the binding of calcium inhibits actin cross-linking by α actinin. Troponin I, part of the complex that regulates skeletal muscle contraction binds calcium via an EF hand and then relieves inhibition of actomyosin interaction (455,90,179).

Calcium binds to calmodulin via EF hand motifs and this calcium/calmodulin complex in turn binds to a number of proteins and regulates their activity (179,455). The complex binds to a number of kinases, among them the multifunctional calcium/calmodulin dependent kinase (CaM kinase II) which, by phosphorylation and the activation of its substrates, regulates the interactions between actin and its associated proteins (455), for example, it abolishes actin filament bundling by synapsin I, and activates myosin light chain kinase which in turn phosphorylates myosin light chains, leading to myosin filament assembly, MgATPase activity and contraction in the smooth muscle cells (455,90,392). Calcium calmodulin can bind reversibly to a number of actin associated proteins and directly hinder or inactivate their actin binding domain. For example calcium calmodulin inhibits actin filament cross linking by fodrin, and on binding to MARCKS inhibits its actin cross-linking but not its actin binding activity (455,168,4).

Actin binding can also be modulated by calcium, via direct binding to a protein that does not contain an EF hand motif. Binding of calcium to a polypeptide chain containing an actin binding domain can induce a conformational change and thus open or activate the actin binding sites, as, for example, in gelsolin, villin and severin (4,455). At high levels of calcium, gelsolin will sever filaments, nucleate filament assembly and cap the barbed end of the actin filament. Reduction in calcium levels inhibits the severing and nucleating activity of this protein, but it will remain associated with the barbed ends of filaments. This gelsolin/actin complex can be dissociated by the interaction of gelsolin with PIP or PIP₂. gCap39 caps the barbed ends of actin filaments in a calcium dependent manner but does not sever them, and the crosslinking activity of L-plastin is disrupted by calcium. The cadherins, in the presence of calcium, mediate cell : cell adhesion and show calcium

dependent resistance to proteolytic degradation (225). The protease calpain II that is enriched in adhesion plaques is activated by calcium. As it cleaves talin it is possible that this is part of the mechanism that regulates adhesion plaque structure in response to cell stimulation (40).

Levels of intracellular free calcium are mediated by the phosphoinositide signalling system, whereby one of the products of PIP₂ hydrolysis, IP₃, released into the cytoplasm, binds to the IP₃ receptor (a calcium channel in a modified portion of the ER surface) and opens calcium channels, thus mobilizing calcium from intracellular stores.(179,392,271) IP₃ may also promote the influx of external calcium by directly activating specific calcium channels in the plasma membrane (27).

1 D CONTROL OF ACTIN CYTOSKELETON BY PHOSPHORYLATION

Protein phosphorylation is one of the most widely used mechanisms for signal transduction, second messengers mediate the actions of agonists by modulating the activity of kinases and phosphatases. Phosphorylation or dephosphorylation of serine and threonine, or tryosine residues results in conformational changes and therefore altered properties in the regulated protein (392,10,194,424,73).

Phosphorylation of some actin associated proteins regulates actin assembly, structure and activity. For example, phosphorylation of caldesmon during mitosis by p34^{cdc2} kinase decreases its affinity for actin, calmodulin and myosin, and might contribute to the reorganization of actin filaments into the contractile ring (494, 239). Phosphorylation of spectrin during mitosis releases it into the cytosol, while protein kinase C phosphorylation of MARCKS inhibits its actin bundling and membrane association and tyrosine phosphorylation of focal adhesion proteins may be an important event accompanying cell adhesion to the extracellular membrane (44, 443). Tyrosine phosphorylation of, for example, paxillin and pp125^{FAK} in adhesion plaques, and the EGF receptor tyrosine kinase stimulates the binding of actin associated proteins with SH₂ domains to these phosphotyrosine containing proteins and induces a cascade of associations between the receptors and actin. Proteins that contain SH₂ domains include src tyrosine kinases, phospholipase C, proteins that regulate the small G proteins ras rac and rho, fodrin and

tensin which is localized to focal adhesions. Binding of tensin to auto-phosphorylated receptor tyrosine kinases via its SH₂ domain may mediate their direct association with the cytoskeleton (443,44). Several regulatory proteins that have SH₂ domains also have at least one SH₃ domain, suggesting that SH₃ domains may also participate in changes resulting from protein tyrosine kinase activity. Almost all known proteins that contain SH₃ but no SH₂ domains are actin associated proteins such as fodrin, cortactin and *Acanthamoeba* myosin 1B. (44).

1 E REGULATION OF ACTIN CYTOSKELETON BY GTP BINDING PROTEINS

Heterotrimeric ($\alpha\beta\gamma$) GTP binding proteins (G proteins) and low Mr monomeric GTP binding proteins, for example the proto-oncogenic ras protein, rac and rho, participate in signal transduction pathways by regulating the activity of specific effectors such as phospholipase C and ion channels, and therefore affect cytoskeletal organization. These molecules can exist in soluble form and therefore interact with cytoplasmic components, such as the cytoskeleton. When bound to GTP, G proteins are active and can recognise and interact with their target molecules. Hydrolysis of GTP to GDP inactivates the G protein, via a conformational change and the interconversion between these two states is regulated by proteins that enhance nucleotide exchange and/or proteins that stimulate their intrinsic GTPase activity (GAP). (222)

Heterotrimeric G proteins are activated by the membrane receptor to which they are bound which enhances nucleotide exchange when a ligand binds to its extracellular domain. Subsequent GTP hydrolysis is determined by the intrinsic GTPase activity of the G protein. Receptor associated G $\alpha\beta\gamma$ dissociates into G α and G $\beta\gamma$ subunits and GTP-G α activates its target, for example an ion channel or a second messenger, such as phospholipase C. The G α -GDP which is released rebinds G $\beta\gamma$ and the trimeric complex reassociates with the membrane receptor.(222)

The activity of human neutrophils, including chemotaxis and phagocytosis following their stimulation is mediated by at least two types of receptor linked G proteins. (19)

Small G proteins require both proteins that increase the rate of nucleotide exchange and GAPs for their GTPase cycle (222). The small GTP binding proteins, rho and rac are

important regulatory or signalling components that are involved in the signal pathway linking growth factors to cytoskeletal organization (69). Stress fibres and adhesion plaque formation is induced in serum starved fibroblasts by microinjection of constitutively activated rho in a manner that mimics serum addition. Rho is thought to mediate the effect of the serum component, lysophosphatidic acid.(69) Conversely, inactivation of rho induces stress fibres and adhesion plaques loss, and cell shape changes. LPA, PDGF and other growth factors may increase the level of GTP bound to rho by inhibiting rhoGAP or activating proteins that increase the nucleotide exchange on rho (366). It is suggested that changes in actin organization, in response to serum and growth factors are dependent at least in part on the activation of rho, although the the precise mechanism of action is unclear (366).

The expression or microinjection of constitutively activated rac in fibroblasts stimulates pronounced membrane ruffling, the accumulation of actin in the membrane ruffles and macropinocytosis, in a manner that mimicks growth factor stimulation. Stress fibre formation occurs after a delay. Conversely, growth factor stimulated membrane ruffling can be prevented by a mutant inhibitory rac, suggesting that rac is also involved in a signal transduction pathway from growth factor receptor to cytoskeletal reorganization. Rac induced stress fibre formation was delayed compared to rho and could be inhibited by inactivation of rho, which is probably acting downstream. It is thought that some growth factors may act through rac to stimulate rho dependent stress fibre formation. Membrane ruffling was unaffected by rho inactivation and is therefore independent of it (365).

V

1 CELL LOCOMOTION

The motility of individual cells, as typified by the movement of fibroblasts across a substrate in culture (2), is vitally important to many fundamental biological processes, such as cell migration in embryonic development, wound healing by epithelial cells and fibroblasts, neurite outgrowth by growth cones and motility of leucocytes at sites of infection and inflammation. Osteoblasts and osteoclasts in bone remodelling move by crawling across the solid substrate in a similar process. (415,427) In disease, the crawling

of malignant tumour cells, that invade and disrupt normal tissue architecture, accounts as much for lethality of cancers as uncontrolled growth (415). Phagocytosis and cell spreading are functionally similar to crawling, while analogous motions transform the shape of blood platelets and enable them to function in the repair of capillaries (415). Crawling is initiated by external stimuli: soluble or substrate bound extracellular molecules binding to the cell surface receptors, or by mechanical distortions, and the transduced signals activate the crawling machinery. To move along a substrate, a cell must adhere to it and the cell margin must spread. One part of the periphery, the leading lamella becomes dominant, and as it extends outwards in the direction of movement the opposing sides retract. To continue polarized locomotion, this extension and retraction of cell surface structures must be controlled temporally and spatially. As it advances, the cell consolidates forward progress by transiently attaching to the cytoskeleton and only those lamellae that adhere to the cytoskeleton are capable of generating locomotion. These cell surface rearrangements depend on the dynamic behaviour of the cortical actin cytoskeleton (415,255). Contractions at the junction of the lamella and the cell body, and also at the rear of the cell, deform the cell body and propel its contents towards the leading lamella. The cell has a polarized appearance, with a flat, broad anterior and narrow tail (415). The advance of lamella proceeds by the extension of lamellipodia/filopodia, that protrude and coalesce, or blebs that expand and then flatten. Membrane ruffles are formed and move back across the lamella. The leading lamella is filled with a dense meshwork of regularly, highly branched, ABP 280 cross-linked actin filaments, which, in fibroblasts, is at least partially orientated with the barbed ends of actin filaments towards the membrane the leading edge. Behind this, longer actin filaments cross at more oblique angles (415,255,428)

Structural actin binding proteins cross link the actin filaments and bind this three dimensional actin gel to the plasma membrane and regulation of the stability of both cross-links and membrane attachment is likely to be important for the local changes in lamellar structure that occur during cell locomotion and spreading (415). A dynamic cycle of actin assembly and disassembly remodels the actin network in the leading lamella and drives cell protrusion. Actin filament assembly at the leading edge, promoted both by nucleating

proteins and the elongation of newly uncapped filaments , and the cross-linking of actin into an orthogonal array by gelation proteins enables the actin network to push against the membrane to form a protrusion (75). Assembly is balanced by disassembly as cross-linked filaments are severed into short fragments and depolymerised into monomers behind the leading edge, thus supplying components for the actin assembly reactions that drive protrusion. In the treadmilling model of actin filament turnover in cell locomotion, actin filaments that are long enough to span the entire breadth of the leading edge are uniformly oriented with their barbed ends at the cell margin. Polymerization occurs exclusively at the cell margin and depolymerization at the rear of the leading edge (427). Forward extension of the leading edge will result if the treadmilling filaments are attached to stationary points of substrate contact . However, in motile fibroblasts, a gradient of actin filament density is seen across the leading edge, suggesting that while polymerization occurs only at the leading edge, depolymerization takes place uniformly throughout its width (427, 176). Observations of actin filament dynamics within the the leading edge of the more rapidly locomoting keratocytes, leads to the nucleation-release model (427,428, 429). In these cells it is suggested that the leading edge contains a meshwork of short actin filaments oriented randomly with respect to polarity, although it may still be at least partially oriented with barbed filament ends towards the membrane. Filament assembly is primarily nucleated at the membrane of the leading edge and filaments are released and crosslinked into a highly dynamic meshwork. Elongation and depolymerisation occurs throughout the meshwork with short filaments disappearing and being replaced by newly nucleated filaments or the elongation of existing newly uncapped filaments. A coherent flux of actin through the leading edge occurs because of the movement of the meshwork as a unit. The cell is driven forward by protrusive activity as long as filament assembly is proportionately greater at the front of the leading edge. Protrusion at the leading edge correlates with an increase in the rate of new filament assembly or polymerisation there. The meshwork disappears at the rear of the leading lamella and this sudden fall in actin density in keratocytes and neurite growth cones is probably due to the activity of capping and severing proteins or a physical barrier to filament progression (427,428, 429). *In vivo*, the behaviour of the actin cytoskeleton is not governed solely by actin polymer dynamics, but also by the effects of a

variety of actin associated proteins - filament severers, barbed end cappers, cross-linkers, nucleators and monomer sequesterers (428).

The nucleation release model of actin assembly has been applied to the propulsion of *Listeria* through the host cell. The short actin filaments that constitute the "comet tail" behind *Listeria* are nucleated at the surface of the bacteria (by profilin), and then released and cross-linked into the meshwork of the tail. In this system, nucleation and elongation occur only at the front of the meshwork, while depolymerization occurs throughout the tail. (428, 432).

A prerequisite for the protrusion of the leading lamella (in fibroblasts) irrespective of the driving force is that either the plasma membrane must detach transiently from its many submembrane actin filament connections, or that membrane bound actin filaments must detach from their neighbours by localized solution of the underlying actin network. While it has been shown that actin polymerization correlates with membrane protrusion, the actual driving force for membrane extension is unclear and a number of hypotheses have been proposed for the production of protrusive forces at the leading edge. One suggestion is that strong actin : myosin contraction at the rear of the cell increases hydrostatic pressure within the cytoplasm so that it is forced towards the front. Localized weakening solution of the cortical cytoskeleton may then allow membrane extension to occur (415,255).

The solution - expansion hypothesis (actin gel swelling) suggests that the transient disruption of the cortical gel by the severing of cross-actin filaments (for example, by gelsolin) induces localized osmotic swelling of the actin gel at the leading edge and this expansion/increased osmotic pressure within the lamella causes it to extend outward and powers the movement of the membrane. Protrusion is directional because the surrounding cortical domains, where the gel remains intact, are resistant to pressure. Subsequent actin assembly and cross-linking reconstitute the gel and the cortex solidifies in the position of osmotically driven advance. This theory is supported by the blebbing and transient thinning of the actin cortex that occurs at the leading edge of migrating cells and the observation that increases in the molarity of the medium retards lamella protrusion.(255,415)

Another possibility is that actin polymerisation at the cell edge directly provides the necessary force for protrusion.

The Brownian ratchet model for membrane protrusion suggests that the membrane at the leading edge, once freed from constraints (actin filament network : membrane binding) fluctuates as a result of Brownian motion. Outward movements provide space for the insertion of actin monomers at the ends of the growing filaments, and a ratchet mechanism drives the cell edge forwards. Actin nucleation proteins (such as ponticulin) are present at the cell edge and promote the formation of new filaments. (415, 428, 269)

Alternatively, membrane associated myosin I molecules may move antegrade along the immobilized actin filaments and push the membrane at the leading edge in the direction of the advance. The extension of the membrane could thus create a space for the addition of actin monomers to the ends of the filaments. Myosin I must be attached to a rigid substrate to exert force and myosin I molecules, bound to the membrane associated cortex, may also facilitate protrusion by movement towards the barbed end of the actin filament anchored to the substrate with actin filaments, bound to myosin I, but not fixed to the substrate, being pushed forward. This filament sliding, however, has not been detected in locomoting cells.(415, 255)

Ruffles form as lamellipodia lift upwards and migrate to the rear of the cell. As the lamella advances, some external molecules on the lamellar surface diffuse randomly in the plasma membrane, while others move toward the leading edge where they remain immobilised and others, especially if cross-linked by antibodies (capping), migrate rearward. Although cortical flow always accompanies cell locomotion, it can be slower than lamellar protrusion and is therefore probably not directly responsible for it. (415)

Filaments growing dorsally and ventrally from the centre of the network mediate cortical flow and substrate adhesion respectively The interaction between myosin II and actin filaments may cause the compression of the lamella at the junctions with the cell body and the tail and may contribute to solation of actin network at the rear of the lamella and in the retracting lamella (415).

For continued locomotion, the rear margin of the cell must retract and in fibroblasts, retraction is preceded by the development of tension between the front and the rear of the

cell. When the tension in the actin meshwork created by actomyosin based contraction exceeds the strength of cell : substrate adhesion at the rear of the cell, attachments are broken and elastic recoil and further contraction cause its retraction. Lamella extension can only proceed if it is attached to the substrate. The continuous formation of cell : substrate contacts at the front of the cells anchors newly polymerized actin to the substrate and resists lamella contractility (255).

The actin assembly, disassembly and cross linking that occurs at the leading edge of motile cells is regulated, both temporally and spatially by actin associated proteins, and these proteins, in turn, may be controlled by polyphosphoinositides and their metabolites.(415,255)

Cell stimulation leads to increases in levels of F actin and cell motility and the signalling pathway may include phosphoinositide metabolism. PIP₂ in the plasma membrane at the leading edge of a motile cell might directly control the timing and location of actin assembly through its interactions with proteins such as profilin and gelsolin, dissociating these proteins from actin and potentiating filament assembly, by the release of monomers and uncapping the barbed ends of actin filament (271,401) and in agonist stimulated macrophages and platelets are shown to gelsolin and profilin redistribute between the cytoplasm and the plasma membrane (455). In some agonist stimulated cells PIP₂ levels increase (417), or possibly are be maintained, as ligand : receptor binding can activate phosphatidyl inositol kinases and lead to increased production of phosphoinositides (415,393), with second messengers being produced instead from PI. Often, however, PIP₂ concentrations are low after chemotactic stimulation, in for example, platelets when actin polymerization from newly generated barbed filament ends is increasing (393,401), and this finding is not consistent with the proposed role of PIP₂ in binding gelsolin and profilin (278). Moreover, changes in actin : gelsolin complexes do not always accompany PIP₂ turnover and actin assembly (401). When PIP₂ is hydrolysed, profilin released from the membrane may promote nucleotide exchange on actin monomers (271), thus enhancing polymerization. Alternatively, PIP₂ may control actin assembly through the production of second messengers, for example, DAG has been shown to induce polymerization and cytoskeletal changes, and increases in DAG concentration correlate with the peak of actin

assembly (401). Levels of PIP₃ also parallel changes in actin assembly, it is not found in unstimulated cells and its levels increase on cell stimulation (500). In addition, it may bind profilin and gelsolin. (278) Other proteins, such as cofilin, ADF, Cap 100 and gCAP 39 also bind polyphosphoinositides and are candidates to regulate phosphoinositide turnover and actin filament assembly (271), making the situation even more complex.

VI

FUNCTIONAL ANALYSIS

1 A FUNCTIONAL ASSAYS

I will describe some of the methods used to study the cytoskeleton, with particular reference to those used in this study.

1 A i Immunofluorescence

Specific fluorescent labelled antibodies against components of the cytoskeleton are widely used to visualize the precise location of their antigens in (249), for example, different stages of the cell cycle (380). These probes can be used either on detergent extracted and/or methanol permeabilized cells or they can be microinjected into living cells. In addition, fluorescent labelled chemical reagents that bind specifically to actin, for example phalloidin, can also be used in double labelling immunofluorescence experiments (461).

Besides their use in the detection of proteins, antibodies can be microinjected into living cells where they may interfere with the function of their antigen, thus providing information about the function of a protein *in vivo*, for example, the inhibition of cytokinesis by microinjection of myosin into starfish embryos.(6) However, non-specificity and cross reactivity can make interpretation difficult (51). Similarly, the microinjection of peptides corresponding to domains of interest such as actin binding domains, can be used in competition arrays.

1 A ii Fluorescence Recovery After Photobleaching (FRAP)

To study dynamic changes in the distribution of a protein over time, for example, in the turnover of subunits in microtubules or actin filaments (330), normal cellular activities must

be maintained. The dynamics of a fluorescent labelled protein, which retains functional competence, after microinjection into live cells and their incorporation into the cytoskeleton can be followed by FRAP. A small area of the cytoskeleton is bleached and fluorescence recovery is followed in the bleached area as fluorescent molecules are incorporated into the dynamic structures (331,186). A photoactivated fluorescent label has also been used to follow the movements of proteins with greater precision. 'Caged' or non-fluorescing molecules, such as caged resorfin labelled actin (429) are microinjected into a cell and after a period to allow for their incorporation, molecules in a selected region are photoactivated with ultra violet light. This method has been used to study actin dynamics in motile fibroblasts (427,229).

1 A iii Negative Stain Electron Microscopy

Negative stain electron microscopy allows the direct visualization of an actin filament complex *in vitro*. The molecules are supported on a thin carbon grid, which is nearly transparent to electrons. Uranyl acetate molecules precipitate onto the carbon grid, except where they are excluded by the presence of the adsorbed macromolecules. The electron beam passes through the protein complex more readily than the surrounding stain, resulting in a negative image (78). Negative stain electron microscopy can provide high contrast surface views of macromolecular complexes/ assemblies, but it is limited in resolution by the size of the metal particles, which can only roughly outline the surface of the molecule. The effect of crosslinking or bundling molecules on actin filaments can be clearly seen, and negative stain electron microscopy has demonstrated for example that at low concentration α actinin cross-links and spaces actin filaments (212) and that fimbrin bundles them into parallel arrays (42)

1 A iv Falling Ball Viscometry

Early studies suggested that actin filaments are cross-linked into gels and that regulation of actin filament polymerization and interactions seemed to be essential for cell motility and the maintenance of cytoplasmic consistency and structure. Falling ball viscometry is a well documented technique used to measure the rapid changes in apparent viscosity that occur as

actin monomers polymerise into filaments and these filaments form networks, or gel, under the influence of ionic strength, cations and a variety of actin associated proteins (352,276). *In vivo*, these proteins are thought to control the formation of actin filament networks, specifying actin filament length, their number and stability and the sites of formation, and to regulate the interactions of actin filaments with each other, the plasma membrane and other cellular structures, such as microtubules (164) Monomer sequestering proteins (cofilin, ADF), capping proteins (gelsolin), severing proteins (fragmin), actin cross-linkers (ABP) and bundlers have all been studied by falling ball viscometry (352,327). Viscosity assays using falling ball viscometers are simple, inexpensive - not requiring any commercial instruments - and highly reproducible. The sample size is small and because the actin monomers polymerize and cross-link within the tube while the sample is at rest, the sample is not disturbed before interaction with the ball, thereby preventing destruction of the gelled samples and preventing the orientation of filaments along the axis of the tube due to flow (350). With appropriate capillary tube and ball bearing diameters, wall effects, such as friction, can be avoided (352). By choosing an appropriate angle of inclination of the tube, the maximum shear rate can be kept low and this enables the study of weak interactions between actin filaments, as the more delicate interactions, such as web formations and T junctions, are conserved. As the system can be adapted a wide range of viscosities can be measured; for solutions of high viscosity, small samples in shorter tubes at a greater angle of inclination would be used, while for those with low viscosity, a longer tube, set at a smaller angle, would give more accurate readings. The experimental conditions, the actin concentration, the presence or absence of a variety of other proteins, ionic strength and the pH of the buffer, can be easily manipulated (352).

Falling ball viscometry measures the apparent, not the absolute, viscosity, because an actin filament network is a complex of non-Newtonian fluid and its absolute viscosity varies with the shear rate. Apparent viscosity is inversely proportional to the velocity of the ball up to the point where the ball will not move. At this point, where the static yield strength is great enough to support the weight of a ball, the yield strength of the sample will be equal or greater than 140 dynes/cm². The limitations of this system are (i) that only two parameters, apparent viscosity and yield strength, can be measured, (ii) for a given sample

the shear rate can only be varied by a factor of ~ 12 using gravitational acceleration and angles of inclination between 10 and 80 degrees, and (iii) even at the low shear rates used, the stress applied to these delicate structures by the falling ball is destructive.(352,350)

1 A v Light Scattering Assay

This assay has been used to measure actin polymerization and the cross-linking and bundling activities of actin associated proteins.(78) Actin filaments scatter light more than actin monomers and the formation of cross-linked networks, by increasing the complexity of a sample, or actin binding by increasing the cross sectional mass of the filaments, increases the amount of light scattered still more. Incident light is directed towards the sample and the intensity of the scattered light is measured at an angle (90°) away from the incident light (78). The advantages of this technique are that it uses native actin and actin binding proteins, the samples are not disturbed by flow/shear, it is rapid, it detects quite small differences and is sensitive to actin binding proteins(78). Parallel electron microscopy studies have shown that the formation of an increased number of actin filament bundles correlates to an increased amount of light scattered by these structures. It has been used to investigate the bundling of actin filaments by HAI/HA vinculin (481), cross-linking/gelation of cytoplasmic actin from cultured cells (228) and binding of tropomyosin to actin (477).

1 A vi Pyrene Labelled Actin/Fluorescence Assays

Pyrene labelled actin is used to measure actin polymerization.(78) The pyrene label changes its fluorescence intensity due to a conformational change as actin polymerizes and , this change can easily be measured in a spectrofluorimeter (232) Pyrene actin is prepared by the reaction of actin monomers with N-(1 - pyrenyl) idoacetamide, a thiol specific reagent, which probably binds to Cysteine 374, the most reactive of the five cys residues in muscle actin, at a maximum stoichiometry of 1 : 1.(79) The assay is very sensitive and, at appropriate wavelengths, the fluorescence of the polymer can be up to 20 times higher than the monomer (78). Native and pyrene actin have been shown to possess identical characteristics with respect to criteria such as the time course of polymerization, elongation

rate constant, intrinsic viscosity and critical concentration and, in addition, they are able to co polymerize (78,79). An advantage of this assay is that the measurements made do not interfere with polymerization kinetics and, because the polymerization reaction occurs in the cuvette, the sample is not disturbed by flow or shear. It has been used, for example, to measure the severing and capping activity of gelsolin (52)

1 B DETERMINING FUNCTION

Amino acid and DNA sequence data have been of great advantage in structure/function studies, since they have revealed, for example, common actin binding domains in the α actinin family of proteins. The function of an uncharacterized protein can be inferred from the conservation of sequences thought to play a role in a specific activity, for example the actin binding domain, the severing domain of gelsolin, villin and severin, and the head domain of myosins in the generation of movement (433).

The study of genetic diseases such as Duchennes muscular dystrophy and haemolytic anaemias, provide additional information on structure : function relationships in cytoskeletal proteins. For example, the defects underlying haemolytic anaemias have been identified as alterations in membrane cytoskeleton proteins, such as protein 4.1 and spectrin, suggesting their role in stabilizing the erythrocyte membrane. (324,139)

However, the unambiguous *in vivo* function of proteins is known in only a few cases and while valuable insights can be obtained from *in vivo* localization studies and *in vitro* functional and biochemical analyses this does not establish cellular function unequivocally. For example, the *in vivo* role of filamin as an actin cross-linker was inferred from its localization with microfilament assemblies in permeabilised cells, and ABP 120 localizes to newly formed pseudopodia and, *in vitro*, forms isotropic gel with actins similar to the cortical meshwork of *Dictyostelium* amoebae suggested a role for ABP120 in the organization of the cortical cytoskeleton. As functions assigned to proteins from their behaviour *in vitro* assays cannot be extrapolated exactly to the *in vivo* situation, *in vivo* functional analysis remains critically important.(48)

A number of approaches have been used to explore cell function directly in living cells: for example, video enhanced microscopy to study cell movements ; microinjection of gelsolin

had little effect on stress fibre organization while microinjection of F actin capping proteins disrupted microfilament bundles (77,124).

Gene transfection and expression of proteins in cells that lack them is a powerful tool for analysing *in vivo* function. For example, gelsolin increased the rate of fibroblast locomotion (87), villin has been shown to induce stress fibre loss and formation of microvilli (122), and α actinin, vinculin, and tropomyosin are known to suppress the tumorigenicity of transformed cells (355,377,145). Mutagenesis of the gene thus leading to the deletion of proteins such as severin (7), ABP 120 (48) or α actinin (324) in *D.discooides* resulted in more or less normal behaviour, but the deletion of filamin resulted in uncontrolled blebbing and loss of directional locomotion in melanoma cells (86). Homologous recombination resulting in the loss of myosin II in *D.discooides* results in normal chemotaxis and phagocytosis but cells were defective in cytokinesis and intracellular particle transport (480), and loss of myosin I produced cells with a delayed chemotactic response and reduced phagocytosis (218). While some results are clear cut such as myosin null mutants in *D. discooides*; the transfection of villin cDNA, (suggesting a role for villin in microvilli organization); and the lethal effects of actin gene disruption in yeast (139), the disruption of some genes thought to have important cellular roles showed little effect on phenotypic behaviour, for example, null severin, α actinin and ABP 120 mutants in *D.discooides*.

These observations have lead to suggestions that either these proteins are not essential for cell motility, or that a considerable degree of redundancy exists, and closely related proteins with duplicated functions can replace one that is eliminated (102). It is not clear why there should be such extensive guarantees for actin based functions, for example more than one crosslinker, or more than one severer, when other more essential functions such as DNA synthesis are not underwritten (38). However, some actin cytoskeleton associated proteins such as vinculin and tropomyosin would appear to be essential, and their function not duplicated, as elimination of vinculin results in a transformed phenotype (114) and restoration of its expression can rescue the tumorigenic phenotype (377), and similarly expression of tropomyosin in transformed cells can suppress neoplastic growth (355). It is possible that in some instances the deletion of a prominently expressed protein might be

benign because the protein has a nonvital function that bestows a small survival advantage on the wild type and this is not detected in optimal/laboratory conditions (102). Exposure of the mutants to unfavourable conditions, such as the forest floor, in the case of *D. discoides*, might indicate that a protein that is not essential in the laboratory may be required for protection against stress in the wild type in its natural habitat (48,40). Absence of observed phenotype changes in mutants in the laboratory may therefore require the development of more sophisticated and innovative assays for cell behaviour. (139,140) Alternatively, loss of some proteins may upregulate compensatory pathways (as has been suggested for inactivation of MyoD which leads to an up-regulation of the myogenic HLH gene *myf 5*)(102) or a system of redundancy may indeed exist to back up essential proteins, with a multiplicity of proteins sharing similar functions allowing for differential control by calcium, and protein cofactors (100). While the similarities in actin regulatory/binding properties suggested by *in vitro* functional assays may be superficial, and reflect limitations in those assays, the *in vivo* roles of these proteins may differ substantially. For example, the actin binding activity of ABP 50/EF1 α or aldolase may be secondary to their function as an elongation factor and a glycolytic enzyme respectively (495,338). . Another possibility is that the expression of selected proteins in some tissues may be functionally superfluous. For example, c-src is expressed in platelets and neurones and while its deletion has no effect these cells, it results in the development of osteoporosis, reflecting its role in osteoclasts. TGF β 1 expression is non vital in embryogenesis, but it modulates the inflammatory response in adults, and knock out of tenascin had no apparent effect in the normal development of the mouse. The cost to the cell of producing small amounts of useless proteins may be minimal and probably less expensive than providing separate control mechanisms for individual proteins, as long as the protein synthetic machinery is not monopolized by the production of superfluous proteins.(102)

Interestingly, *D discoides*, deficient in both α actinin and ABP120 are not defective in several major cellular functions - growth, cell motility or chemolaxis, phagocytosis and pinocytosis, but development/morphogenesis is greatly improved and normal development is resumed when a functional α actinin gene is introduced and expressed, indicating that

morphogenesis requires at least one actin cross-linking protein and the loss of two will disrupt the normal development (483,324) Thus actin cytoskeleton based functions may depend on the combined activity of actin binding proteins as a whole, with each type of cell movement driven by a specific blend of actin binding proteins (38)

VII

TRANSFORMATION

In normal cells *in vivo*, cell division, cell growth and cell movement are highly controlled. On transformation, these normal constraints are lost, the cells proliferate and may form tumours. Transformation can result from mutations that occur spontaneously or be induced in cell culture by exposure to radiation, carcinogenic chemicals, tumour promoters such as TPA , or by infection with DNA or RNA viruses.

1 A SIMIAN VIRUS 40 (SV40)

The SV40 DNA virus infects a variety of cultured mammalian cells but only cells from primates are permissive hosts (107). The virus normally remains autonomous and is not stably incorporated into the host genome, but in a permissive host it multiplies lytically and kills the cells (107,5,89). Permissive cells, on infection, enter the S phase of the cell cycle and provide the virus with the replication enzymes necessary for viral DNA synthesis. Proteins encoded by the viral (early) genes activate host DNA replication enzymes which the virus uses to replicate its own genome and synthesize the products of late viral genes and the host cell then lyse and releases viral particles. Infection of a nonpermissive host results in abortive transformation, only the early transforming genes are expressed, and induction of S phase does not lead to viral DNA replication. The host cells proceed through the cell cycle continuously and the viral genome is degraded or lost by dilution whereupon the cell reverts to normal (107,5, 89).

Occasionally the SV40 genome integrates into that of the host cell , where it is replicated with the host genome (integration occurs without an obvious preference for a particular site on either the host or viral DNA), or it forms a plasmid that undergoes controlled replication without killing the host. The early transforming viral genes are constitutively expressed and

the continuous activity of the viral gene products, large T and small t antigen, establish and maintain the transformed phenotype, and drive the cell through the cell cycle (107).

Small t antigen (Mr 20kDa) is a cytoplasmic protein and is required for full transformation. It may regulate protein phosphatase 2A.(107)

More than 90-95% of large T antigen (Mr90kDa) is localised in the nucleus where it occurs as a free nucleoplasmic protein or is bound to either DNA or the nuclear matrix. It plays an important role in DNA transcription and replication. The remaining 5-10% is cytoplasmic, and is associated with and may be integrated into the plasma membrane and exposed on the transformed cell surface. It can be phosphorylated on threonine and serine residues and has protein kinase activity. Large T expression is essential and sufficient to initiate the change of phenotype since it (a) stimulates cell proliferation as measured by induction of DNA synthesis in quiescent cells, (b) induces cell transformation, (c) immortalizes primary cells and (d) causes tumorigenicity. The specific binding of large T to DNA does not appear to be important for transformation, but its interactions with tumour suppressors, such as the retinoblastoma susceptibility (RB) gene product, and its related cellular protein p107, and the p53 gene product are believed to play decisive roles in transformation. (107)

1 B ROUS SARCOMA VIRUS (RSV)

RSV is an RNA retrovirus. In the normal life cycle, a retrovirus enters the cytoplasm of a host cell, where it synthesizes a double stranded DNA copy of its genome with reverse transcriptase. This DNA migrates into the nucleus, circularizes and integrates into the host genome where it acts as a template for the translation of mRNA for transcription of viral products and genomic RNA for the next generation of viral particles. The viral genome, although stably linked to that of host, does not seriously damage the host. The genetic information encoded by RSV is replicated as part of the host DNA and is distributed to daughter cells where it is transcribed and new viral particles are made. Thus proviral DNA can continually direct the synthesis of new viral progeny without killing the host cells. Sometimes the viral genome becomes integrated into the germ cell line, and is inherited by the offspring of the host. The retroviral genome normally encodes proteins that do not affect the growth characteristics of the host. Those viruses that can transform the host cell

may contain additional oncogenic genetic material, appropriated from normal host DNA. The normal product of an oncogene, expressed under normal controls, may play an important role in fundamental cell processes such as cell division or cell growth, but the oncogene product may be expressed constitutively, at higher levels, or in a non cell or tissue specific manner, because of loss of part of the gene which regulated protein expression or activity. Thus the oncogene encoded protein may be either qualitatively or quantitatively changed. Integration of retroviral DNA near a proto-oncogene, part of the host genome, can bring its expression under new controls, so that it becomes a transforming oncogene (5,89).

The oncogene responsible for the transforming ability of RSV, which is unnecessary for its own survival or reproduction, is the viral src oncogene, v-src. It is derived from c-src and encodes a 60 kDa protein, pp60^{v-src}, which is similar to the c-src gene product but has a different C terminus (217). The only known function for pp60^{v-src} is that of a protein tyrosine kinase, and its unrestrained activity in phosphorylating cellular substrates on tyrosine residues is presumed to be responsible for the initiation and maintenance of the diverse phenotypic changes that are characteristic of transformed cells (217,41). Cells transformed by RSV have a ten fold higher level of phosphotyrosine than normal cells (217). pp60^{v-src} is itself phosphorylated on serine and tyrosine residues by cAMP dependent protein kinase and autophosphorylation respectively. While a small population is associated with intracellular membranes, more than 80% of is associated with the cytoplasmic face of the plasma membrane (via myristylation of its N terminal amino acid residues) and this membrane attachment is required for efficient transformation (224). pp60^{v-src} is enriched in regions of cell : substrate the invadopodia of transformed cells (443), and its transforming activity of correlates with its degree of association with the detergent insoluble cytoskeleton (489,224) Although several actin associated proteins, that are components of the adhesion plaque, such as vinculin, talin, paxillin, integrin, pp125^{FAK}, tensin (397,384,489,224,165) are phosphorylated on tyrosine by pp60^{v-src} in transformed cells in parallel with microfilament bundle breakdown, reorganization of adhesion plaque components and changes in adhesion characteristics, there is no absolute correlation between phosphorylation of any of these targets and disruption of the

cytoskeleton, and the mechanism by which the RSV tyrosine kinase oncogene induces the reorganization of the actin cytoskeleton is poorly understood.(111, 224)There appears to be little selectivity of substrate for pp60 v-src and a number of adhesion plaque proteins and other intracellular proteins (such as MAPs, glycolytic enzymes and calmodulin 144) exhibit increased levels of protein phosphotyrosine to a greater or lesser extent following its activation. The stoichiometry of phosphorylation of paxillin and cortactin (up to 30%) suggests that this may be significant (489,443), but similar phosphorylation of paxillin occurs in a nontransforming mutant fibroblast line (193). While tyrosine phosphorylation in the cell correlates well with cytoskeletal and adhesion plaque disruption, it may reflect a saturation of the cells normal regulatory mechanism. It is suggested that tyrosine phosphorylation is an important event that accompanies attachment rather than disruption of extracellular matrix : cell interactions, as high concentrations of phosphoproteins are present in the adhesion plaque of nontransformed cells and the invadopodia of transformed cells contain tyrosine phosphoproteins such as paxillin, cortactin, tensin, pp125^{FAK} (489,384,165). Furthermore, inhibition of kinase activity blocks the formation of invadopodia and matrix degradation, suggesting a functional link between phosphorylation and invasion. Phosphorylation of the cytoskeleton in transformed cells may therefore be important for adhesion since the invadopodia attaches to the extracellular matrix prior to degrading matrix components (193,443).

1 C THE TRANSFORMED PHENOTYPE

Oncogenic transformation causes a number of changes in cell morphology, biochemistry and cell behaviour including:

1. Loss of density dependent inhibition of cell division (increased saturation density) so that cells grow to unusually high densities or loss of growth arrest capacity (484)
2. A decreased requirement for endogenous growth factors, possibly because transformed cells produce and release their own (transforming) growth factor requirement (425), paralleled by an increased expression of transforming growth factor receptors (217)
3. Loss of anchorage dependent growth, enabling transformed cells to grow in soft agar or suspension (217,425)

4. Loss of contact inhibition, so cells will grow beyond confluency and in multilayers (217)
5. Increased glucose transport and metabolite uptake which may explain the ability of transformed cells to grow at low serum levels (217)
6. Increased membrane fluidity, and therefore rapid aggregation of surface receptors by lectins (89)
7. Reduction in surface fibronectin (345)
8. Increased production and secretion of proteases, for example plasminogen activator,(449) and down regulation of protease inhibitors (28) resulting in an increase in proteolysis of ECM components.
9. Altered translation/transcription (217)
10. Dedifferentiation (425, 441)
11. Immortalization of cells, which continue to proliferate indefinitely (484)
12. Ability to produce tumours *in vivo* (217)
13. Altered expression of certain cell adhesion molecules to allow invading cells to migrate through tissue and blood vessels (345,93)

1 D EFFECT OF TRANSFORMATION ON THE ACTIN CYTOSKELETON

A striking change is often seen in the actin cytoskeleton with large scale disruption of microfilament bundles as actin filaments fail to organize into stress fibres and become diffusely distributed throughout the cytoplasm and/or concentrated into the cortical meshwork (251). This loss of stress fibres is neither due to a significant change in the relative proportion of G and F actin, (174)(since there is no apparent net depolymerization of actin filaments) nor to changes in the total actin content of transformed cells, compared to their normal counterparts (158), but rather to a reorganization of the polymeric actin from stress fibres to other filamentous forms within the cell (111). This change from well organized, cross-linked actin bundles containing up to 10 filaments to an extensive, convoluted meshwork of mainly single filaments with multiple, random interconnections, that may be only weakly associated with the plasma membrane (111) is accompanied by the altered expression and/or activity of some actin associated proteins, which sever, crosslink,

stabilize actin filaments or to anchor them to the plasma membrane. The loss of thick filament bundles in 3T3/SV40 and hamster adenovirus transformed cells correlates with an increased thickness of the cortical actin network, and the cytoarchitecture of these cells resembles that of rapidly moving or dividing fibroblasts (472).

The time course of cytoskeletal changes can be divided into two stages, early events resulting from reorganization of F actin filaments lead to the transient appearance of ruffle-like "flowers", which contain actin, α actinin, tropomyosin and myosin, on the dorsal surface of the cell concomitant with the appearance of short actin filaments and detachment of actin filaments from the plasma membrane. This is followed by the disappearance of actin stress fibres. (32, 111) The reduction in actin filament bundles is paralleled by a decrease in the number and size of cell : cell contacts and adhesion plaques in RSV transformed cells. F actin, α actinin, vinculin, talin, fimbrin, paxillin, tensin, cortactin and pp60 v-src become aggregated into a cluster of small, round patches or rosettes, on the ventral cell surface partly underneath the nucleus (91). These invadopodia are sites of close contact with the substrate, (59,91,489) where the cells attach, degrade and penetrate the extracellular matrix (443,193).

Alterations in transformed cells that may be linked to the disruption of stress fibres and redistribution of actin filaments include anchorage independent growth, loss of contact inhibition (217) excessive blebbing of the plasma membrane (86) increased membrane ruffling (313), increased mobility of cell surface glycoproteins, a marked reduction in the number of adhesion junctions and a change in the architecture of these structures, with the formation of new cell processes such as invadopodia (91,193) loss of adherence to both the substrate and other cells, which may induce the change to a rounded morphology with few well defined processes, and the increased motility of transformed cells (452).

Biochemically these cell shape changes are associated with specific alterations in the expression/synthesis of a small number of proteins, including actin and some actin associated proteins. While the total amount of actin in transformed cells remains more or less constant, the predominant actin isoform may change. For example, accumulation of the α smooth muscle actin isoform, which is abundant in normal stress fibres and is associated with a flattened, well spread, contact inhibited phenotype, and the mRNA that encodes it, is

greatly inhibited and may be totally down regulated in transformed NIH 3T3, Rat 2, Ref 52 and mouse L (SV40) cells (252). Changes may also occur in the ratio of γ : β cytoplasmic actin expressed in transformed cells, for example, while normal rat 2 cells have less γ than β , following spontaneous transformation they express more γ than β , and dramatic changes in the cell morphology of transformed HOS (Human osteosarcoma derived) cells (MNNG HOS) are paralleled by a 50 % reduction in the synthesis of β actin (253). A variant form of β actin, designated A^x , which is distinct from α , β and γ actin and is derived from β actin by a single amino acid mutation, is expressed in human fibroblasts that have been transformed by chemical carcinogens (HUT 14) (456,251). While this mutated actin retains polymerization competence (although it is incorporated at a slightly lower rate into cytoskeleton of HUT 14 cells, 315), it appears to distribute differently (456). The different affinity of actin associated proteins, for example tropomyosin for β or γ compared to α actin, may contribute to changes in microfilament properties (61). Actin isoform expression can also affect the expression of associated protein isoforms (386). Thus, the production of variant actins which may be polymerization incompetent, changes in actin expression and disturbances in the balance of isoforms are likely to be associated with the rearrangement of microfilaments, changes in cell shape and motility and may lead to an increase in the tumorigenic potential of cells.

In addition, the product of the transforming oncogene (*fgr*) which encodes both the N-terminus amino acid sequence of actin and a tyrosine specific protein kinase, may interfere with the organization of the cytoskeleton by blocking actin polymerization or by phosphorylation of cytoskeletal targets that are normally inaccessible to the kinase activity (315).

1 D i Actin as a Proto Oncogene

Proto oncogenes are responsible for the control of fundamental cell processes and, if they are disrupted, may induce transformation. It has been suggested that the actin gene may be a proto oncogene (25). It has been suggested the actin filament network may be involved in anchorage dependent growth and proliferation in normal cells, and that microfilament disruption may be involved in the transforming process, although some transformed cell

types (for example, certain endothelial cells) maintain their stress fibre network (25). For example, initiation of DNA synthesis in response to EGF is blocked if actin filaments are disrupted with cytochalasin B, and is sensitive to the degree of cell spreading in adherent cells (23), and in many transformed cells loss of anchorage dependent growth control correlates with disruption on the actin cytoskeleton (484,25). Synthesis of actin relative to other proteins is specifically inhibited in anchorage dependent fibroblasts cultured in suspension, and is greatly increased in reattaching cells (17,108) Actin gene expression is down regulated on terminal differentiation and there is a dramatic and rapid increase in its transcription (in G₀ to G₁ /S transitions) in response to growth factors or TPA, an expression pattern mimicked by *c-myc*, *c-myb* and *c-fos* proto oncogenes, whose nuclear located products may be concerned with transcriptional control (25). In addition a mutant actin is expressed in the transformed cell line HUT 14 (456), and α actin isoforms are down regulated on transformation (252)

The mechanism of action of the putative actin proto-oncogene in regulating cellular processes is unknown, but growth factor (PDGF/EGF) receptor kinase activity and the tyrosine kinase activity of the pp60^{v-src} product of src oncogene induce rapid reorganization of the actin filament cytoskeleton. Growth control messages might be conveyed to the nucleus via a physical link of stress fibres, perhaps perhaps leading to the nuclear lamins, and the transcriptional machinery of the nucleus (25). It has been suggested that information may be transferred from the cell surface via the cytoskeleton to the nucleus and result in the exposure of a particular set of genes at the nuclear periphery (359). In transformed cells loss of differentiation properties and growth control due to altered gene expression correlate with cytoskeletal changes. Alternatively, a change in the polymerization status of actin may alter the G : F actin ratio in the nucleus, which might alter its effects on transcription.

1 E EFFECT OF TRANSFORMATION ON ACTIN ASSOCIATED PROTEINS

The disruption of microfilaments in transformed cells is accompanied by the down regulation of a small number of actin associated proteins, including tropomyosin, non muscle caldesmon, myosin light chain 2, gelsolin, actin binding protein, α actinin,

schwannomin, MARCKS, thymosin β 4 and α calponin. More than one protein in a cell may be down regulated, for example, in MRC-SV2 compared with normal MRC5 cells, gelsolin, actin isoforms, α actinin and tropomyosin are all down regulated. The altered expression of proteins associated with cell : cell and cell : substrate contacts, such as vinculin, integrins, catenins and cadherins, and the phosphorylation of cytoskeletal proteins are also thought to contribute to the establishment and maintenance of the transformed phenotype.

1 E i Tropomyosin

In cells transformed by both DNA and RNA viruses, the expression of at least one of the three major high Mr tropomyosins (which have a high affinity for α actin and are associated with stress fibres) is reduced or completely down regulated, at the level of transcription, while the levels of one or both of the low Mr isoforms (which bind weakly to actin and are associated with both stress fibres and β actin enriched actin filaments in the peripheral motile regions of cells) are unchanged or increased (294,253). The total amount of tropomyosin in transformed cells is reduced in comparison with their normal counterparts, where sufficient tropomyosin is synthesized to saturate actin binding sites (355). Tropomyosins are thought to stabilize actin filaments by regulating their interactions with other associated proteins, such as filamin, villin, gelsolin, a 55 kDa protein, DNase 1 and ADF, and may have a role in the organization and maintenance of stress fibre bundles. The function of a reduction in tropomyosin levels might be to expose sites on the actin filament to the action of severing or depolymerising proteins (457). The differential expression of tropomyosin observed in transformed cells, which may be effected in part by the altered expression of actin isoforms (386), correlates well with observed morphological changes and may be involved in or cause the disruption of stress fibre bundles and thus be responsible in part for the tumourigenicity of transformed cells (294). Transfection of cDNA encoding one tropomyosin is able to reverse, in part, the transformed phenotype of cell (355). Recently it has been shown that expression of a 3' untranslated region of tropomyosin mRNA could also function as a tumour suppressor, inhibiting anchorage independent growth and invasiveness of neoplastic cells, suggesting that untranslated mRNAs have sequences with important functions in regulating cell growth (361)

The acute transforming oncogene of human colon carcinoma (trk or onc D) encodes a hybrid protein with the N terminus amino acid sequence of nonmuscle tropomyosin and an unknown receptor with tyrosine kinase activity. This raises the possibility of transformation either by interference with the formation of the normal cytoskeleton or by phosphorylation of normally inaccessible cytoskeletal targets, as trk is redirected from its normal position and/or kinase substrate near the plasma membrane by the presence of the tropomyosin sequence (25) Furthermore, it is suggested that the transforming effect of trk oncogene may be due not only to its kinase activity, but also because the tropomyosin 3' untranslated region is missing from trk mRNA (361)

1 E ii Non Muscle Caldesmon

Non muscle caldesmon is down regulated by 66% in transformed cells (S7-1) and whereas in normal cells it is localised on stress fibres (coincident with tropomyosin) and the ruffling membranes of fibroblasts, in RSV transformed cells this distribution becomes more diffuse (337) In addition to its role in regulating the motility and/or contraction of non muscle cells, caldesmon is thought to increase the stability of stress fibres by binding and bundling actin filaments itself, and by promoting the actin binding activity of the low Mr tropomyosin isoforms and increasing the actin binding of the high Mr tropomyosin isoforms.

Tropomyosin, in turn, is able to enhance caldesmon : actin binding. The actin binding activity of caldesmon and its stimulation of tropomyosin:actin binding are regulated by Ca^{2+} /calmodulin, and in transformed cells the levels of Ca^{2+} /calmodulin increase, so that even the small amount of caldesmon expressed in these cells may be unable to either bind to actin or enhance the binding of tropomyosin to actin. The actin filaments, therefore, are not so highly cross linked and lose their protection against the activities of proteins such as gelsolin. The down regulation of caldesmon expression in transformed cells may therefore be involved in, or cause, the instability of stress fibres and may correlate with the loss of Ca^{2+} regulation seen in transformed cells (337, 492).

1 E iii Myosin Light Chain 2

Phosphorylated smooth muscle myosin light chain 2, in the presence of Ca^{2+} /calmodulin, increases actin activated myosin ATPase activity, and is therefore critical for the

contraction process in these cells. In non-muscle cells the phosphorylation of myosin light chain 2 also induces the assembly of myosin II into ordered bipolar filaments, which, with actin, form the main component of the stress fibre network. In human fibroblast cell lines there are at least 3 isoforms of myosin light chain and the dramatic changes in cell shape and cytoarchitecture following transformation may be caused in part by the complete down regulation, at the level of transcription, of a smooth muscle myosin light chain isoform, as seen in transformed HOS fibroblastic cells. Interestingly, this parallels the down regulation of the smooth muscle specific α actin isoform in these cells (234)

1 E iv Gelsolin

Gelsolin is thought to control the assembly of actin in the cortical cytoplasm in response to Ca^{2+} and polyphosphoinositides. It is found both at the cell periphery where it contributes to the conversion of actin from a gel to a sol, and on fine filaments throughout the cytoplasm. There is a positive correlation between gelsolin expression and both a flat cell morphology and an ordered growth pattern.

Gelsolin is down regulated in transformed cells (457), and interestingly, undifferentiated and proliferating cells also have low levels of this protein (238). The disruption of the actin filament network and the increased motility that is associated with the acquisition of the transformed phenotype might be accounted for, at least in part, by the down regulation of this very abundant actin regulatory protein which appears to have an important role in the organization of the cortical cytoplasm, chemotaxis, cell motility and cell division (457). Functionally, the increased levels of Ca^{2+} in transformed cells may activate the small amounts of gelsolin that are expressed. This would lead to the formation of shorter actin filaments and the increase in cell motility. It is hard to see how a reduction in the amount of gelsolin can account for the disruption of the cortical actin network, a paradox that may be resolved by experiments that determine the time course of gelsolin down regulation.(170).

1 E v Actin Binding Protein 280

By cross linking actin filaments into an isotropic network and connecting them to the plasma membrane via its association with a glycoprotein, ABP 280 stabilizes the cortical cytoplasm and therefore indirectly the cell surface in normal cells. In transformed cells ABP 280 is down regulated, either at the level of transcription or because the mRNA encoding it

is unstable, and this may affect the cortical cytoplasm of these cells and lead to a reduction in the provision of attachments for membrane bound receptors. Three unrelated tumour cell lines, derived from human malignant melanomas, which lack ABP 280 have no large actin filament bundles, show impaired locomotion because they are unable to localize their protrusive activity in response to stimulation, and show apparently uncontrolled, extensive and continuous surface blebbing, which is the result of peripheral cytoskeletal instability. The expression of ABP 280 in these cells after transfection of the cDNA encoding it restores efficient and directional motility and reduces blebbing (86).

1 E vi Thymosin β 4

Thymosin β 4 is a abundant protein in normal cells where it sequesters actin and thus regulates actin polymerisation. While it is abundant in non metastatic cell lines, thymosin β 4 is down regulated in some metastatic cell lines, and it has been suggested that its expression is correlated with the metastatic potential of, for example, colorectal carcinomas. It is possible that the down regulation of thymosin β 4 would result in a loss of regulation of actin polymerization, leading to alterations in cell locomotion, chemotaxis and cytoarchitecture and finally an increase in metastatic capacity (491,152).

1 E vii MARCKS

Transformation by viral oncogenes, such as v-src, is accompanied by the down regulation of MARCKS expression, probably at the level of transcription. Dephosphorylated MARCKS is important in maintaining the flattened morphology of normal non-transformed cells, possibly by bundling and anchoring actin filaments to the plasma membrane, while phosphorylation of MARCKS by protein kinase C may lead to the dissociation of actin filaments from the plasma membrane and the acquisition of a more rounded cell shape. The reduced levels of MARCKS in, for example, mouse tumour cell lines, coupled with the increased levels of phosphorylation observed in v-src transformed cells may contribute to the morphological changes that occur upon transformation. Inhibition of v-src protein tyrosine kinase activity restored levels of MARCKS mRNA and protein to normal levels, suggesting that down regulation of MARCKS in transformed cells may be the direct result of persistent v-src kinase activity (215, 487, 216).

1 E viii Calponin

The lower Mr isoform of calponin has been found to be down regulated in benign tumours of human urogenital tract (leiomyomas)(98)

1 E ix α Actinin

α actinin cross links actin filaments and therefore increases their stability, and is largely localized to cell junctions where it is involved in the attachment of actin filaments to the plasma membrane. In these adhesion plaque regions, α actinin binds to actin, vinculin, zyxin and integrins, and therefore is likely to have a role in the organization of cytoskeleton, cell adhesion and cell motility. The interactions between the cytoplasmic components of adhesion junctions are highly co-operative and efficiently recruit components from the soluble pool. Alterations in the equilibrium of adhesion plaque components between the membrane bound and cytoplasmic fractions can result in the assembly or disassembly of adhesion plaques. It is possible that a reduction in the amount of α actinin in transformed cells may lead to disassembly of adhesion plaques and therefore the disruption of the stress fibres. Loss of its bundling activity in stress fibres may also lead to their instability. For example, SV40 transformed 3T3 cells (SV T2) express 6 fold lower levels of α actinin than normal 3T3 cells. Transformed cell lacking this protein were rounded, able to grow in suspension culture and to form tumours *in vivo*.

Transfection of the gene encoding α actinin into transformed cells where its expression is down regulated, results in the expression of normal levels of α actinin and suppression the transformed phenotype, leading to cells with a flatter morphology, a decreased ability to grow in soft agar and a marked reduction in tumorigenic ability (145).

1 E x Schwannomin/Merlin

Schwannomin has a striking similarity to members of the moesin/ezrin/radixin family of proteins which are associated with actin and are concentrated at cell : cell and cell : substrate junctions, cleavage furrow, microvilli and ruffling membranes, suggesting that they have an important role in the tight association of actin filaments with the plasma membrane. Inactivation of the gene encoding schwannomin causes neurofibromatosis 2, which is characterised by the development of central nervous system tumours (schwannomas and meningiomas) and therefore it has been suggested that schwannomin (and maybe other

family members) may act as tumour suppressors by affecting actin filament/plasma membrane associations (441,437,370). The loss of stable cell : cell, cell : matrix interactions may lead to increased migration, cell shape changes and loss of contact inhibition.

1 F EFFECT OF TRANSFORMATION ON PROTEINS OF ADHESION JUNCTIONS

Proteins which interact indirectly with actin as part of a cascade but are vitally important for the integrity of the stress fibre microfilament system, are the components of cell : cell and cell : substrate junctions. A number of these are also down regulated or altered in transformed cells.

1 F i Vinculin

Vinculin is a major structural component of cell : cell and cell : substrate contacts in normal cells. In certain malignant cells of fibroblast and epithelial origin the levels of vinculin are markedly reduced or undetectable and these cells adhere poorly, lack cell : cell and cell : substrate adhesion sites and have a reduced number of microfilament bundles, suggesting that the loss of vinculin from adhesion junctions (which form a bridge between the extracellular matrix and actin based cytoskeleton) may well lead to the disruption of adhesion junction integrity and therefore to the disarray of microfilament bundles, diminished cell adhesion, altered morphology, and increased cell motility seen in transformed cells (114). Expression of vinculin following transfection of a cDNA encoding this protein in malignant mouse carcinoma cell lines, which express low levels of vinculin, suppresses the transformed phenotype of these cells and results in an increase in substrate adhesiveness, organized microfilament bundles, a decreased ability to grow in soft agar, decreased locomotory activity and a reduced capacity to develop tumours *in vivo* (113). The vinculin encoded by the transfected cDNA is incorporated into cell : substrate adhesion sites and may promote the cooperative assembly of focal contact proteins to form an increased number of stable adhesion sites. (113,377). Conversely, normal 3T3 cells transfected with antisense vinculin mRNA leads to development of the transformed phenotype. (114)

1 F ii Integrins

Integrins are transmembrane proteins that bind extracellular matrix components and link them to the actin cytoskeleton via the proteins of adhesion plaques, and therefore mediate cell attachment and cell spreading. Alterations in integrins, therefore, could lead to the altered adhesion, migration, motile activity, morphology and cytoskeletal organization that are characteristic of transformed cells and associated with malignancy (93).

Transformation of rat NRK Nil8 cells by RSV, or mouse sarcoma viruses encoding ras oncogenes, leads to alterations in integrin expression with a marked reduction in the levels of the high affinity fibronectin receptor, $\alpha_5\beta_1$, the loss of two other integrins of unknown ligand specificity, $\alpha_A\beta_1$ and $\alpha_B\beta_1$, while $\alpha_3\beta_1$, a polyspecific receptor (binding fibronectin, laminin and collagen) is retained (345). The integrins in these transformed cells are not aligned with actin microfilaments, but are diffusely distributed over the cell surface, reflecting the disruption of the adhesion plaques. Consequently, these cells have a reduced ability to assemble the fibronectin-rich extracellular matrix and a diminished ability to adhere to fibronectin. They will attach to fibronectin coated substrates, but require higher levels than their normal non-transformed counterparts which may reflect the lower affinity of $\alpha_3\beta_1$ for fibronectin. Transfection of cDNA encoding integrin $\alpha_5\beta_1$ can suppress the tumourigenic phenotype of these cells (177).

Since integrins and adhesion plaques are also the sites of signal transduction, it is possible that alterations in integrins leads to the disruption of signal pathways (193).

1 F iii Cadherins

E-cadherin (uvomorulin or L-CAM) is expressed in most normal cells and is a transmembrane protein that binds to identical proteins in neighbouring cells in the presence of Ca^{2+} . It is localised to, and important for the maintenance of cell : cell contact sites, and therefore the differentiated state. The cadherin complex is linked to the actin cytoskeleton via α catenin and other plaque components such as vinculin (452) The actin cytoskeleton is tightly associated with the plasma membrane through its association with components of these cell : cell junctions. Down regulation, or functional inactivation by loss of contact with the cytoskeleton, of E-cadherin (463) has been observed in transformed cells, such as colon carcinomas and epithelial cells transformed with Harvey and Moloney sarcoma virus

(463) or RSV (340). Down regulation of cadherins is a critical step in the promotion of epithelial cells to an invasive phenotype, as it leads to a weakening of cell : cell adhesion which enables a malignant cell to detach from a primary tumour (340) Loss of E cadherin in cultured carcinoma cells is associated with an altered morphology, and an increase in motility and invasiveness, and correlates with the disorganization of the actin cytoskeleton (340, 463, 28). Transfection of the cDNA encoding E cadherin into a highly invasive epithelial tumour cell line will suppress the malignant phenotype, and the cells become partially differentiated rather than dedifferentiated. Conversely, normal MDCK cells will become invasive if cell : cell adhesion is inhibited by anti-cadherin antibodies. (137,28,463)

1 F iv α Catenin

Expression of α catenin (which is associated with cadherins in cell: cell adherens junctions) is down regulated in a human prostate cancer cell line and a human lung cancer cell line, which although they expressed normal cadherins at the cell surface, showed reduced aggregation activity, and therefore a poorly differentiated, rounded morphology. Transfection of cDNA encoding α catenin into lung cancer cells restores cell aggregation activity and results in the production of a differentiated epithelial cell sheet. (441)

1 G SYNERGY AND REDUNDANCY

The down regulation of a selected group of cytoskeletal proteins appears to parallel the onset of neoplasia. In normal cells some or all of these proteins may act synergistically to organize and control actin assembly and architecture (457). Alterations in the expression of one or more might therefore be directly or indirectly involved with the acquisition of the transformed phenotype. For example, the high Mr tropomyosins that are down regulated on transformation protect actin filaments against the severing activity of gelsolin and villin, the depolymerising activity of DNase 1 and ADF, and inhibit binding of gelsolin, spectrin and α actinin (262). This effect is enhanced by nonmuscle caldesmon which also enables low Mr tropomyosins to similarly stabilize actin filaments. Thus, the loss of high Mr tropomyosins and caldesmon from transformed cells may contribute to cytoskeletal reorganization (492).

In addition, caldesmon itself can cross link actin and actin : myosin and stabilize actin filaments, and its actin binding is enhanced by tropomyosin. The reduction in caldesmon levels in transformed cells, in conjunction with increased levels of calmodulin, calcium and a 55kDa actin binding protein which inhibits the binding of low Mr tropomyosins, and replacement of high Mr with low Mr tropomyosins may result in the destabilization of stress fibres and cause, in part, the morphological changes seen in transformed cells (492). Modifications in the balance of actin isoforms expressed in transformed cells to which actin binding proteins may bind with different affinities may also be associated with the reorganization of the actin cytoskeleton. Changes in nonmuscle gene expression can affect not only the organization and distribution, but also the expression of actin associated protein isoforms, for example, a decrease in β : γ actin ratio correlates with the down regulation in synthesis of the higher Mr tropomyosins (386).

The loss or modification (by calpain proteolysis, for example) of one or more proteins from the adhesion plaque, which may depend on the cooperative binding of all components and the integration of multiple low affinity interactions that occur between its components for its integrity, may in part lead to the instability, and therefore the altered size, architecture and abundance of adhesion plaques that is seen in transformed cells, and hence cytoskeletal reorganization (443).

It is possible that the changes in microfilament organization accompanying transformation may, in some cases, involve the coordinated down regulation of several components, rather than a single protein (457,355) reflecting the synergistic nature of their organization and control of the actin cytoskeleton (457) This may also reflect the redundancy of some of these proteins, although in several cases, the loss of only one protein (for instance, elimination of vinculin or tropomyosin with antisense mRNA) induces transformation, and the transfection and re-expression of a single protein in a transformed cell can rescue the transformed phenotype (113,377,355). It is possible that the alteration of one protein may in some instances provoke a cascade of effects that culminate in cell transformation. Interestingly, it has been found that the expression of a 3' untranslated region of mRNA encoding tropomyosin could suppress anchorage independent growth and tumour formation (that is act as a tumour suppressor) suggesting that the transformed effect might

be the result of both loss of a structural cytoskeletal protein and loss of growth control mediated, in some so far unknown manner, by its mRNA (361)

While changes in actin cytoskeletal architecture may be effected by the direct action of an oncogene (for example pp60 v-src) on a group of actin associated proteins, which might either alter their activity or induce their down regulation (for example the continual phosphorylation of MARCKS by pp60 v-src is thought to cause its down regulation,215,487), the reorganization of actin in RSV transformed cells also correlates with activation of a phosphatidyl inositol kinase (111) and generation of DAG and PIP₂. Similarly, microinjection of phospholipase C into quiescent NIH 3T3 cells (12), or expression in cells of a 7 membrane spanning receptor coupled to PLC leads to their transformation (27), suggesting that alterations in phosphoinositol metabolism that occur on transformation may promote or induce alterations in the microfilament cytoskeleton via interactions with proteins such as profilin, gelsolin and α actinin.

1 H MICROTUBULES AND INTERMEDIATE FILAMENTS IN TRANSFORMED CELLS

In many cell types transformed by DNA or RNA tumour virus, there is a decreased number of microtubules and tubulin and therefore a more diminished microtubule network. This may be due to the increased levels of calmodulin in transformed cells depolymerizing microtubules, and leading to a reduced number of microtubules and even reduced tubulin synthesis by an autoregulation mechanism. (25) The MAP2 mediated interaction between microtubules and intermediate filaments is disturbed by transformation with RNA tumour virus such as RSV and it has been suggested that pp60^{src} may act on the components of the linkage between the microtubules and the intermediate filaments (25).

The pattern of differential expression of genes encoding various intermediate filament proteins during embryogenesis, as well as in adult and in tumour formation and metastasis, are well established and because many metastatic solid tumours retain a specific intermediate filament type expression characteristic of the parent primary cells, antibodies to various intermediate filament proteins can be used as a probe for cell typing in tumour diagnosis, including metastatic tumours(225). For example, cytokeratin synthesis is

usually maintained in malignantly transformed epithelial derived carcinomas, even if they have lost their epithelial structure; gliomas are identified on the basis of GFAP expression; muscle sarcomas express desmin; non-muscle sarcomas, lymphomas and leukaemias express vimentin; and tumours originating from the sympathetic nervous system express neurofilaments (25, 236). Changes in cell shape from a well spread flat morphology to a more rounded one induced by suspension culture resulted in a dramatic reduction in vimentin expression in normal and malignant cells and therefore alterations in the organization and/or the expression of vimentin in response to cell shape change may well be related to metastatic behaviour (25,24).

VIII

METASTASIS

In vivo, many solid tumours are benign, that is, while they have many of the features of the transformed phenotype, for example, loss of differentiation, growth control and contact inhibition, they are only locally invasive (373) and do not spread throughout the host. (340) They can be successfully treated by chemotherapy, radiotherapy or surgery. Some tumours, however, become malignant, due to their ability to develop secondary growths (or metastases) in organs distant from the site of tumour origin. This invasive, metastatic spread of tumours is one of the commonest causes of death in cancer patients (340, 373).

Metastasis is a multistep process, requiring distinct interactions of metastatic cells and the regulation of a particular set of gene products .

1. The dissociation of individual cells from the primary tumour
2. Invasion from the site of primary tumour into the surrounding host tissue/epithelial basement membrane to reach the interstitial stroma/connective tissue
3. Entry into the circulatory system (intravasation) by invasion of the basement membrane and endothelial cell layer of the blood capillary wall
4. Dissemination via the blood or lymphatic system
5. Arrest in a specific organ

6. Penetration of the vascular endothelium and/or its underlying basement membrane (extravasation)

7. Invasion from the perivascular stroma into the organ parenchyma at the site of metastasis

8. Proliferation, and neovascularization

(373,340,452,110)

The release of proliferating cells from the primary tumour requires reduction or loss of adhesion to their neighbouring cells and the substrate. This may be mediated by the down regulation of cell :cell adhesion molecules such as E-cadherin, α catenin, and the down regulation of other cell : cell adhesion molecules, leading to the loss of functional desmosomes (452,31,340). Loss of specific integrins, for example the fibronectin receptor, $\alpha 5\beta 1$, can reduce adhesion to the extracellular matrix in invasive cells (198) *In vitro*, in comparisons of pairs of tumour cell variants with differing metastatic potential it was found that those with low potential had extensive, well developed substrate contacts and defined networks of actin filament bundles, while those that were highly metastatic attached only weakly to the substrate, had fewer and smaller adhesion plaques and a disorganized actin cytoskeleton. (25,362)

To invade host tissue metastatic cells must adhere to, selectively degrade and migrate through a proteolytically modified extracellular matrix (452,263,93). The extracellular matrix can both obstruct and assist metastasis by serving as a barrier to invading cells and supporting migration of such cells by allowing their transient adhesion (452) Modification of the extracellular matrix must be controlled, ensuring digestion of some components to enable invasion, while preserving the substrate required for migration (457). Temporally and spatially regulated changes in the secretion of proteases (and protease inhibitors) modifies the extracellular matrix and leads to the mobilization of tumour cells, induction of migration pathways in the host tissue, and the release and/or proteolytic activation of growth, angiogenesis and motility factors, specific factors which promote invasion and metastasis (28) In addition to the production and secretion of proteases by the tumour cells themselves, normal host cells can be actively recruited for this purpose. Adhesion to the modified basement membrane surface is mediated by receptors expressed on the tumour cell surface and changes in integrin expression and therefore cell : matrix adhesion can

affect the traffic of tumour cells for example, the novel expression of $\alpha_v\beta_3$ integrins correlates well with invasive activity in melanomas, and $\alpha_2\beta_1$ expression (a collagen/laminin receptor) (93,452,226) increases in aggressive melanomas and may be related to the ability of these cells to 'rework' collagen as they migrate. CD44 also acts as an extracellular matrix receptor (Hyaluronic acid /collagen) and is modified in tumour cells, its expression correlates with the metastatic potential of a rat carcinoma cell line (452).

Tumour cells can also modulate the by secretion of structurally modified extracellular matrix components and/or deposition of abberant amounts of extracellular matrix molecules. Expression of fibronectin is down regulated in parallel with the loss of the high affinity fibronectin receptor integrin $\alpha_5\beta_1$. They can also stimulate host cells to influence the extracellular matrix nondegradatively by, for example, the secretion of tenascin which may act as an anti-adhesive molecule in fibronectin interactions.

By acting in concert, these alterations contribute to the increased migratory behaviour of invasive cells through the extracellular matrix.(93,226,452,345)

Following intravasation cells are disseminated through the host via the blood or lymphatic system. The circulatory anatomy plays a role in the dissemination of tumour cells (373,452,340) and cells circulating in the vascular system are arrested either by mechanical trapping in the capillary bed, which may be potentiated by their interaction with host platelets, (nonspecific arrest) or via a specific interaction between a tumour cell receptor and glycoproteins expressed by endothelial cells (addressins/selectins) and/or the exposed basement membrane (198). *In vitro*, different malignant cell lines will preferentially attach to endothelial cells of their *in vivo* target organ (373, 316). A normal counterpart of this site specific homing of metastatic cells into organs is the adhesion of activated lymphocytes to vascular walls (and their subsequent entry into tissue) which is dependent upon specific adhesive interactions between lymphocytes and selectins expressed by endothelial cells (198). Endothelial cells may retract on lymphocyte adhesin, exposing the more adhesive basement membranes. Several human tumour cell types express the $\alpha_4\beta_1$ (VLA4) integrin, which enables normal lymphocytes to adhere to VCAM-1 on the surface of endothelial cells (particularly upon stimulation by cytokines such as interleukin 1 and tumour necrosis factor). In addition, two selectins, E-selectin and INCAM 110 have been shown to

mediate the adhesion of a human colon carcinoma cell and a human melanoma cell line respectively. (373).

In the circulatory system tumour cells form contacts with various host cells. Their interaction with platelets may protect them from immune or mechanical killing, provide additional adhesive mechanisms and serve as a potential source of growth factors. Cell : cell adhesion molecules of the immunoglobulin superfamily, including carcinoembryo antigen (CEA), deleted in carcinoma (DCC), MUC18 and ICAM-1 mediate the adhesion of tumour cells to other host cell types. ICAM-1, expressed by human melanoma cells, interacts with the leukocyte integrin LFA-1 and may allow an interaction between tumour cells and leukocytes which, rather than promoting immune killing of the tumour cells, appears to contribute to their dissemination and protect them from physical stress. (452,340) To enable tumour cells to evade recognition and killing by cytotoxic T cells, intravascular tumour cell clusters and metastatic lesions of human carcinomas generally show decreased expression of MHC class I antigens. (452, 340)

Having adhered to endothelial cells, the arrested malignant cell can cause their retraction and expose the basement membrane allowing the tumour cells to penetrate the vascular endothelium and /or the underlying basal lamina and invade the tissue where proliferating cells form secondary tumours. While some tumours can invade passively through cell growth and expansion, active cell motility enhances their metastatic potential and is a property of most invasive tumour cells (31) . The successful growth of solid tumours depends on their vascularization, and transformed cells induce angiogenesis by the synthesis and secretion of factors that stimulate normally quiescent endothelium to develop new capillaries, a process which also involves cytoskeletal remodelling (200, 263). For example, the switch from a normal cell-associated state of β FGF to its extracellular release in fibrosarcomas is concomitant with neovascularization and tumorigenicity (221)

The changes from a normal to a metastatic phenotype are complex but the underlying mechanism causing their aberrant behaviour may be associated with activation and repression of only a few genes and the differential expression of their products.

Finally, it is interesting that invasion and metastasis involve many of the same processes exhibited by normal cells during development. The motile behaviour and cellular invasion

of tumour cells is similar to the cell movements observed in migrating fibroblasts and epithelial cells in wound healing, activated lymphocytes at sites of inflammation, osteoclasts during bone remodelling and many cell types that show developmentally controlled migration, involving the formation of cell processes at the leading edge, regulation of cell surface receptor/ligand binding and coordinated mobilization of the cytoskeleton. However, while, for example, angiogenesis by normal endothelial cells and metastasis are functionally similar, they differ in their regulation (263) thus transformation may induce re-expression of genes normally only during embryogenesis (28,340,452,198) Many of the properties of malignant cells, important for their metastatic tumorigenic potential, such as loss of contact inhibition, enhanced motility, cell : cell and cell : substrate contact involve activation, remodelling and/or perturbation of the cytoskeleton. The importance of cytoskeletal alterations in the invasive phenotype is clearly demonstrated in studies where transfection of proteins associated with actin, and important in maintaining the stability of the actin cytoskeleton, such as tropomyosin (355), α actinin (145), vinculin (113), integrins (345) and cadherin (28) suppress the tumourigenic phenotype of transformed cells in which expression of these molecules is down regulated.

The monoclonal anti - protein C4 antibody used in this investigation was raised by Dr D Lawson using the following protocol.

The immunogen was prepared by the method of Feramisco and Burrige (112). Fresh chicken gizzard was homogenized in a Waring blender with 3 x 10 s bursts at top speed in 10 vol of deionized water (4°C) + 0.5mM PMSF. The suspension was centrifuged at 5,000 g for 10 min, the supernatant discarded, the pellet resuspended as above and blended for 10 s at low speed. The centrifugation was repeated and the pellet resuspended at 37°C in 10 vol of 2 mM Tris, 1 mM EGTA, 0.5mM PMSF (pH 9 at room temperature), and stirred for 30 min at 37°C. The centrifugation was repeated and the supernatant adjusted to pH 7-7.2 with 0.5M acetic acid. 1M MgCl₂ was added to 10mM. After stirring for 15 min at room temperature the suspension was centrifuged as above and the supernatant used as the immunogen. BALB/c mice were immunized with 100µg of immunogen per injection : day 1, subcutaneous in complete Freund's adjuvant; days 14, 63 and 77, intraperitoneal in incomplete Freund's adjuvant; day 81, tail bleed and test for antibody response; days 84, 85 and 86, intravenous injection; day 87, fusion.

The fusion was performed as described, using the myeloma cell line SP2 (245).

When the hybridoma cells were two thirds confluent the supernatants were tested by immunofluorescence on formaldehyde fixed methanol permeabilized 3T3 cells. Antibody producing wells were cloned by limiting dilution into 96 well plates, with a feeder layer of adherent BALB/c macrophages at a concentration of 5 x 10³ cells/ml. Cloning was carried out twice and cells producing anti-protein C4 stored in liquid nitrogen.

METHODS

1. TISSUE CULTURE

A. Media and Plastics

i. Media

The media routinely used in these experiments were E4 low bicarbonate or RPMI (+ penicillin, 100 units/ml and streptomycin, 0.01%), supplied by ICRF and stored at 4°C. Unless otherwise indicated, media was supplemented with 10% foetal calf serum (FCS) (Gibco BRL, Northumberland Biologicals, Flow Laboratories or Imperial Laboratories) which had previously been heat inactivated at 56°C for 30 minutes to destroy complement, aliquoted and stored at -20°C. Versene [0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline, (140mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.5mM KH₂PO₄, Sigma Chemicals), (PBS)], stored at 4°C, and trypsin (0.25% in tris saline), stored at -20°C, used for cell passaging were also provided by ICRF and were used at a ratio of 4:1 respectively.

ii. Plastics

Cells were cultured in 25 cm², 75 cm², or 175 cm² tissue culture flasks (Falcon). For suspension culture experiments, cells were cultured in 10cm diameter tissue culture petri dishes (Falcon), smeared with high vacuum silicon grease (Beckman Instrument Inc. or Dow Corning Corp.) where appropriate. For immunofluorescence studies, cells were passaged and then cultured in 24 well dishes (Falcon), with each well containing a UV light sterilised 13mm diameter glass coverslip (BDH). To raise high titre antibody stocks, antibody secreting hybridoma cells were cultured in Falcon round bottom 96 well plates (see section C). The centrifugation steps necessary for tissue culture were carried out in 30 ml universal tubes (Sterilin).

B. Cell Lines and Secondary Cell Cultures

Table 1 Cells used in these investigations

Line/name	Origin/species	Cell type	Supplier (see appendix)
3T3	mouse	fibroblast	ICRF
3T3 SV40	mouse	transformed fibroblast	ICRF
REF	E15 rat embryo	fibroblast	
Rat 1	rat	fibroblast	S. Kellie
Rat 1 RSV	rat	transformed fibroblast	S. Kellie
Rat 1 RSV LA29	rat	temperature sensitive transformed fibroblast	S. Kellie
Butlers	human	fibroblast	Flow laboratories
Detroit 98	human	epithelial	Flow laboratories

C. Cell Passaging and Storage

i. Adherent Cells

The medium from adherent cells was removed from the flask by aspiration, cells were rinsed briefly with 4:1 versene/trypsin which was discarded and replaced with 5 mls of fresh versene/trypsin. The flasks were incubated at 37°C until all cells had dissociated from the substrate (usually 5 mins), transferred to universal tubes containing 15 mls of E4 medium +10% FCS to inactivate the trypsin, spun at 200g for 5 minutes, resuspended in fresh medium and counted in a Neubauer haemocytometer. Cells were plated at a

concentration of 2.5×10^3 - 2×10^4 per ml for coverslips, 5×10^3 - 5×10^4 for flasks depending on the experiment and cell type used. Cell culture flasks were maintained at 37°C in 100% humidity and 5% CO₂.

ii. Non-adherent cells

Cells were resuspended in their growth medium, transferred to universals, centrifuged as above and plated at a concentration of 1×10^4 - 1×10^5 cells/ml in fresh medium.

iii. Suspension Cell Culture

To prevent the cell adherence of fibroblasts in culture, REFs were plated at a concentration of 1×10^6 /ml onto petri dishes which had previously been treated with high vacuum silicon grease. This grease had previously been tested for any effect on cell viability and found to be non-toxic with 97-100% of cells reattaching and spreading normally after 72 hours in suspension culture. Cells were cultured in suspension for up to 72 hours, then they were either returned to normal, adherent culture, or prepared for either immunofluorescence (section 2Ai) or SDS-PAGE/Immunoblotting (section 3Aia).

For some experiments, cells were returned to adherent culture, in the presence or absence of actinomycin D ($2.5 \mu\text{g/ml}$) or cycloheximide ($25 \mu\text{g/ml}$) (Sigma) (83,172). At these concentrations adherent cell viability was > 95%, as determined by trypan blue exclusion.

Cells were harvested from suspension culture as for non-adherent cells in section 1Cii.

iv. Cell Storage; Freezing and Thawing.

All cells were stored under liquid nitrogen (-196°C) in FCS plus 10% dimethylsulfoxide (DMSO) (Sigma, cell culture grade). Prior to freezing, cells were counted, pelleted at 200g for 5 minutes, and resuspended in FCS/DMSO at 4°C at a concentration of 1×10^6 cells/ml. 1ml aliquots were transferred to 1.5 ml screwtop freezing vials (Nunc)

which were insulated and cooled slowly to -70°C over a 24 hour period. This significantly improved cell viability.

Cells were thawed rapidly in a 37°C waterbath, until the medium was liquified, transferred to a universal containing 20mls of E4 at 4°C , pelleted at 200g for 5 minutes, resuspended and plated as above (section 1C).

D. Rat Embryo Fibroblast (REF) Production

Secondary REFs were used for all of the experiments in this study. These were derived from 14-15 day old embryos which were dissected and rinsed twice in an excess of E4. Using sterile scissors and forceps, the hind and fore limbs were removed, (care being taken to avoid the tail), placed onto a sterile glass slide, teased apart by the shearing action of two 21 gauge needles (Sabre) until no lumps of tissue remained, and transferred to a 175 cm^2 flask containing E4 + 10% FCS. These were incubated overnight, the growth medium replaced, and the flasks then left for about five days until the dividing fibroblasts were almost confluent. The cells were passaged, maintained in flasks, frozen for storage, or plated onto coverslips (1C). Although cells remained viable for at least 20 passages, they were routinely discarded after 10.

2. IMMUNOFLUORESCENCE

In order to study cytoskeletons using immunofluorescence, various protocols were used to fix, permeabilize and stain cells growing as monolayers on glass coverslips.

A. Fixation

i. Methanol Fixation

Coverslips of cells were rinsed in PBS, plunged into methanol at -20°C (BDH Analar), incubated at -20°C for 10 minutes, rehydrated in 6 changes of PBS and blocked in freshly made block buffer (0.3% bovine serum albumin (BSA, Sigma fraction V), 100mM lysine to block any reactive aldehyde groups, in PBS) for at least 10 minutes at room temperature.

ii. Formaldehyde/Acetone Fixation

This protocol was recommended by Molecular Probes Inc for use with the F-actin probe, fluorescein-phalloidin, which they supplied. Coverslips were rinsed in PBS, incubated in 3.8% formaldehyde (BDH Analar) in PBS for 12 minutes at room temperature, rinsed in 6 changes of PBS, plunged into acetone at -20°C , incubated at -20°C for 10 minutes, rehydrated in 6 changes of PBS and blocked as detailed in section 2Ai.

B. Detergent Extraction

i. Extraction

For initial experiments cells were permeabilized in 0.1% triton X100 in 50mM KCl, 4mM MgCl_2 , 1mM EGTA, 10 mM imidazole pH7, 1mM NaN_3 (Sigma) for 5 minutes at 4°C . For subsequent experiments, coverslips of cells were rinsed briefly in Buffer P \pm 60mM KCl (12 mM K Phosphate buffer pH 6.8, 2mM MgCl_2 , 1mM EGTA, 1mM ATP, Sigma), extracted at 4°C for various times (from 15 seconds to 15 minutes) in Buffer P \pm 60mM KCl + 0.5% CHAPS (3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propansulfonate, Boehringer Mannheim), rinsed in Buffer P \pm 60mM KCl, plunged into methanol at -20°C , then rehydrated and blocked as above. Control coverslips were rinsed briefly in Buffer P + 60mM KCl, plunged into methanol, rehydrated and blocked as above.

ii. Permeabilized Cell System

To rebind transgelin to permeabilized cells the following protocol was used. REFs were detergent extracted for 15 minutes at 4°C in 2mls of Buffer P + 60mM KCl + 0.5% CHAPS, rinsed in Buffer P + 60 mM KCl, blocked for 5 minutes at room temperature in 3% BSA, 100mM lysine in Buffer P + 60 mM KCl, and then incubated at 4°C for 30 minutes in transgelin (1.5 or 3 μM), in either Buffer P \pm 60 mM KCl in a total volume of 60 μl . Coverslips were then rinsed/plunge-fixed in 7 ml of 1% glutaraldehyde (Fluka) for 30 seconds at 4°C , rapidly transferred to 2% formaldehyde (BDH) in PBS for 5 minutes at room temperature, rinsed briefly, incubated in 1 mg/ml sodium borohydride in PBS at 37°C for 10 minutes to block free aldehyde groups, rinsed in PBS and blocked as above.

C. Immunofluorescence Labelling of Cytoskeletons

Coverslips of permeabilized, fixed cells, after blocked for 10 minutes in PBS + block buffer, were transferred to a humid chamber and 50µl of mouse anti-protein C4 antibody at a concentration of 50µg/ml in PBS + block buffer added for 30 minutes at room temperature. Coverslips were then rinsed 6 times in 20 mls of PBS, once in PBS + block buffer, and incubated for 30 minutes with 50 µl of goat anti-mouse IgG specific, conjugated to rhodamine (Cappel Laboratories), at a concentration of approximately 50 µg/ml. Coverslips were rinsed in 6 times in 20 mls of PBS, blotted and mounted onto glass slides in 20% gelvatol (Polyvinyl alcohol, supplied by Monsanto Polymers, made up in PBS). For double immunofluorescence where fluorescein reagents were also used the anti-fade Citifluor was used instead of gelvatol. For phalloidin/fluorescein labelling cells were fixed as detailed in section (2Aii), antibody labelled as above and then incubated for 5 minutes at room temperature in a 1:10 dilution of phalloidin/fluorescein in PBS/BSA, rinsed and mounted as above.

D. Antibodies

i. Monoclonal anti-protein C4 antibody

The mouse anti-protein C4 secreting hybridoma was raised from a fusion performed by D. Lawson (245). The immunogen was a partially purified fraction from chicken gizzard (112). The class of anti-protein C4 antibody was determined by double diffusion (Ouchterlony) and was found to be IgG₁.

Two methods were used to obtain high titre stocks of anti-protein C4 antibody. First, anti-protein C4 antibody secreting hybridoma cells were plated in 96 wells at a concentration of 1×10^5 cells/well, and cultured for up to 10 days. The growth medium, containing the antibody, was then collected by pipetting, spun at 3,000 g for 5 minutes, millipored (0.45 µm pore, Sartorius filter), NaN₃ added to 16mM and stored at 4°C. This supernatant was used neat in immunofluorescence and for labelling immunoblots. The advantage of this method is that there are no contaminating mouse immunoglobulins in the antibody.

Secondly, ascites tumours were raised as follows. 5×10^6 hybridoma cells secreting anti-protein C4, were washed in 20 mls of RPMI and resuspended in 0.5 mls RPMI, and injected intraperitoneally into female Balb/c nude mice. Ascites formed within 7-10 days, and mice with well developed ascites were sacrificed, the ascitic fluid withdrawn from the peritoneum by syringe and 21 gauge needle, mixed with mouse blood to enhance clotting, phenylmethylsulfoxyl fluoride (PMSF, Sigma) added to 5mM and NaN_3 to 0.1mM, left to clot at room temperature for 4 hours, and then at 4°C overnight. The clot, containing most of the erythrocytes, was removed, the remaining fluid centrifuged at 10,000 g for 5 minutes, the supernatant removed, aliquoted and stored at -70°C , or passed over a Sepharose protein A column to remove contaminating, non-specific mouse IgG (section Dii).

ii. Purification of Monoclonal Anti-Protein C4 Antibody

2g of Sepharose 6MB protein A (Pharmacia), was swollen in 0.1M Na Phosphate buffer pH6.8 ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}/\text{Na}_2\text{HPO}_4$), 5mM NaN_3 overnight, centrifuged at 200 g for 2 minutes, washed 2x in Na Phosphate buffer, loaded into a 15 x 1.5 cm Econocolumn (Biorad), (final column volume 10 mls), washed with 2 column volumes of Na phosphate buffer, and the baseline OD_{280} recorded. The capacity of the protein A for Ig is up to 9 mgs/ml. 4 mls of ascites containing approximately 10 mgs of total IgG was adjusted to pH 8.6 with 1M Tris base (Sigma), and loaded onto the Protein A column at a flow rate of 5 mls/hr. The column was washed down to baseline absorbance with 0.1M sodium phosphate buffer, the bound antibody eluted with 0.1M Na Acetate pH6 ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$, Sigma, brought to pH6 with glacial acetic acid) and the peak collected using an LKB Multirac 2111 fraction collector connected to an LKB uvicord S11 spectrophotometer. This pH value was chosen since it is known to specifically elute $\text{IgG}_{1,0}$. The column was cleaned with Na Acetate pH 3.5, re-equilibrated with Na Phosphate buffer, 5mM NaN_3 and stored at 4°C . Fractions containing the purified antibody were pooled, dialysed against PBS, NaN_3 added to 16mM and the antibody aliquoted and stored at -70°C . The usual yield was about 1 mg purified anti-protein C4/ml crude ascites. This antibody was used for immunofluorescence at a concentration of $50\mu\text{g/ml}$.

Table 2 Protein A Purification

Fraction	OD ₂₈₀
1	0.063
2	0.034
3	0.026
4	0.034
5	0.095
6	0.371
7	1.68
8	2.15
9	2.1
10	1.55
11	0.95
12	0.485
13	0.254
14	0.138
15	0.086
16	0.52
Background	0.032

iii. Polyclonal Antisera

Rabbit anti-transgelin polyclonal antibody was raised by D.Lawson and myself. A partially purified fraction of sheep aorta (see section 4B) was dialysed overnight into 50mM Tris base pH 7.5, electrophoresed on a 3mm thick 12% SDS PAGE preparative gel (see section 3Aii), with molecular weight standards (Gibco BRL) and purified transgelin (see section 4B) run as standards. The gel was lightly stained with Coomassie Blue, destained(see section 3Aii), and the band containing transgelin excised, rinsed in Tris base pH 6.8, and cut into small pieces. These were loaded into plastic tubes, (which had been

sealed at one end with 1cm of 2% agarose 10 (BDH) in distilled water), and sealed into the tubes with 1% agarose in SDS running buffer (section 3Aii). The protein was electroeluted from the gel pieces at 50 volts constant volts for 16 hours and collected in dialysis tubing (Spectra/Por, 6-8000 molecular weight cut-off). 4 volumes of acetone at -20°C were added to precipitate the protein from the pooled eluates, and left to stand for 30 minutes in an ethanol/dry ice bath. The precipitated protein was collected by centrifugation at 20,000 g for 30 minutes, the supernatant discarded, pellets allowed to air dry, then resuspended in a minimum volume (usually 0.5mls) of 0.1M MOPS (3-[N-Morpholino] propane sulfonic acid, Sigma) pH7.5, and dialysed against the same buffer for 48 hours. Protein concentration was determined by reading OD₂₈₀, the solution concentrated if necessary by filtration with Millipore filter units (Mr 10,000 cut off), NaN₃ added to 16mM and the protein stored at 4°C prior to injection in rabbits. To prepare the immunogen for injection, the protein, PBS and Freund's adjuvant were mixed by forcing them through a 19 gauge needle using a 1ml glass syringe until an emulsion had formed. This was tested by placing a drop onto water; if the drop did not disperse over the surface of the water, an emulsion had formed. The times chosen for the immunization schedule shown below have been established as maximising the immune response in rabbits (169).

Table 3 Immunization Schedule

Time in days	Injected material	Route of Injection
0	250µgTg/0.5ml CFA	Sub-cutaneous
22	250µgTg/0.5ml CFA	Sub-cutaneous
31	Test Bleed 5mls	
43	250µgTg/0.5ml ICFA	Sub-cutaneous and intramuscular
52	Ear bleed 20 mls	
60	250µgTg/0.5ml ICFA	Sub-cutaneous and intramuscular
73	Ear Bleed 15-20 mls	
80	250µgTg/0.5ml ICFA	Sub-cutaneous and intramuscular
90	Bleed 30 mls	
201	500µgTg/0.5mls ICFA	Sub-cutaneous and intramuscular

216	500µgTg/0.5mls ICFA	Sub-cutaneous and intramuscular
225	Bleed 20mls	
230	400µgTg/0.5mls ICFA	Sub-cutaneous and intramuscular
240	Bleed out	

CFA-Complete Freund's Adjuvant

ICFA-Incomplete Freund's Adjuvant

Tg-Transgelin, in a total volume of 0.5mls of PBS

Blood was allowed to clot at room temperature for 4 hours, the clot was then ringed by passing a glass rod around the inside of the tube, and left at 4°C overnight. NaN₃ was added to 5mm. To maximize yields, the clot was suspended over a filter funnel on a glass spool. The clot was removed and the serum centrifuged at 10,000 g for 10 minutes, heat treated at 57°C for 30 minutes to inactivate complement, centrifuged as above and tested by immunofluorescence on methanol fixed REFs. (See section 2Ai, 2C). Albumin was removed from positive sera (after day 52) by adding saturated ammonium sulphate solution to a final concentration of 40% with gentle stirring for 15 minutes at 4°C, incubation at 4°C for 2 hours, and centrifugation at 20,000 g for 15 minutes. The supernatants were removed, the pellets resuspended in 20mM Na Phosphate buffer pH 7.5 and dialysed against this buffer for 3 days, with multiple changes of buffer.

To obtain an IgG fraction, DEAE 52 (Whatman) was used. A slurry of gently packed, wet DEAE was added to the ammonium sulphate fraction at a ratio of 6-7 mls packed DEAE : 80 mgs protein, and stirred for 30 minutes at room temperature, transferred to a Buchner funnel in a side arm flask and the excess supernatant removed by gentle suction. The DEAE was washed with several bed volumes of 20mM Na Phosphate buffer, the filtrate decanted and retained, and the DEAE washed sequentially with 20mM Na phosphate buffer + 30mM then this same buffer + 70mM NaCl. These DEAE cuts were collected separately, dialysed overnight into PBS, their protein concentration determined by reading

OD₂₈₀, concentrated if necessary by filtration with millipore filtration units, and the various fractions adjusted to the same concentration and tested by immunofluorescence on methanol extracted REFs (see section 2Ai,2C). The 30mM NaCl cut when tested gave the strongest and most specific immunofluorescence labelling and was therefore used for all subsequent studies.

E. Immunofluorescence Reagents.

Immunofluorescence reagents used for this study were obtained commercially.

Fluorescein phalloidin (Molecular Probes Inc) was used at a dilution of 1:5000.

Goat anti-mouse IgG conjugated to rhodamine (IgG class specific antisera) goat anti-rabbit conjugated to fluorescein and goat anti-mouse Ig (polyspecific), were purchased from Cappel. The lyophilised antibodies were rehydrated with sterile distilled water, allowed to stand at 4°C for 2 hours to completely solubilize, centrifuged at 148,000g for 20 minutes to remove aggregates, aliquoted, stored at 4°C or -20°C, and used at a dilution of 1:100, approximately 50µg/ml.

F. Disruption of the Cytoskeleton with Cytochalasin D and Colchicine

REFs were incubated at 37°C with 1µM cytochalasin D (Sigma) for 15 minutes before fixation with formaldehyde/acetone (2Aii) and labelling with anti-protein C4 / goat anti-mouse IgG-rhodamine and fluorescein phalloidin, or 1µM colchicine (Sigma) for 2 hours before fixation and labelling with anti-protein C4 / goat anti-mouse IgG-rhodamine and rabbit anti-vimentin / goat anti-rabbit -fluorescein. The distribution of protein C4, actin filaments and intermediate filaments were compared.

G. Microscopy and Photography

Microscope slides were viewed using a Nikon Optiphot microscope, a Nikon x 60 objective and a x10 eye piece. Immunofluorescence was performed with a Nikon epifluorescence attachment. Cells were photographed using T-Max film (Kodak) and a Nikon 35mm camera. Exposure time was determined with a Nikon spot metering apparatus. Breakthrough filters (B2E, Excitation 450-490, Barrier 520-560 and G2A, Excitation

510-560, Barrier 590, Nikon) were used for fluorescein/rhodamine doubles to prevent rhodamine breakthrough into fluorescein, and vice versa. T-Max was developed in HC110 (Kodak) at 20°C, as recommended by Kodak. All photographs were printed on Kodak resin coated paper, grades 1-5, (depending on the contrast requirement) and developed in Dektol, as recommended by Kodak.

3. BIOCHEMISTRY

A. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

i. Sample Preparation

a. Cells

All cells used for these experiments were cultured as described in previous sections (1C). Cells prepared for biochemistry were cultured until almost confluent, harvested by (a) trypsinization (section 1Ci), (b) scraped off with a rubber policeman, or (c) pipetted off for either cells in suspension or non-adherent culture. Cells were transferred to a 1.5 ml microfuge tube or a 30 ml Universal, pelleted by centrifugation (10,000 g for 30 seconds at room temperature), resuspended and washed 3 times in PBS containing 1mM PMSF, 0.1µg/ml soya bean trypsin inhibitor (SBTI) and 5mM EDTA. The final cell pellet was resuspended in 2 volumes of Laemmli sample buffer (2.3% SDS, 2% glycerol, 90mM tris pH 6.8, 0.005% bromophenol blue and 120mM 2-mercaptoethanol) (Ref), and boiled for 3 minutes before freezing at -20°C or -70°C.

b. Tissue

Tissue was dissected, chopped into small pieces (2mm² approx) with scissors, scalpel and forceps, washed as for cells, boiled in 2 volumes of Laemmli sample buffer and stored at -20°C or -70°C.

c. Proteins

Dilute protein samples were concentrated by a variety of methods prior to SDS-PAGE.

i. Acetone Precipitation

Protein samples were precipitated by the addition of 4-5 volumes of acetone at -20°C, allowed to stand in a methanol/dry ice bath for 30 minutes (or overnight at -20°C) and collected by centrifugation at 30,000 g for 1 hour. The supernatant was discarded, the tubes dried carefully with Kimwipes without disturbing the pellets which were allowed to air dry prior to resuspension in Laemmli sample buffer, boiled for 3 minutes and stored at -20°C or -70°C.

ii. Methanol/Chloroform Extraction

4 volumes of methanol/chloroform (mixed in a ratio of 4:1) were added to the protein sample, mixed by vortexing, 3 volumes of distilled water added, vortexed, and centrifuged at 10,000 g for 1 minute at room temperature to separate the aqueous and solvent phases. The upper aqueous layer was removed with a pasteur pipette, leaving the organic phase and the interphase, where the protein banded in the tube. To this, 3 sample volumes of methanol were added, vortexed and centrifuged at 10,000 g for 5 minutes at room temperature. The supernatant was removed and the protein pellet allowed to air dry before being solubilized in an appropriate buffer or sample buffer, and processed for SDS-PAGE as above.

ii SDS-PAGE

Two separate sizes of gel system were used. First, 10 or 12% acrylamide gels of 17 x 13 cm and either 0.7 or 1.5 mm thick were run using a BRL V16 vertical run apparatus. This gel system was also used for 5-15% acrylamide gradient gels which were poured using a Biorad linear gradient pourer. Secondly, I used a Biorad Miniprotean II vertical electrophoresis system, for 8 x 6 cm, 0.7mm thick, 12% acrylamide gels. The gel and buffer system used were based on those described by Laemmli. The gels incorporated 5-15% acrylamide (BDH Electran), 0.14-0.4% N-N-Methylene bisacrylamide (BDH Electran), 0.37 M Tris base (pH 8.8) (Sigma), 0.05% ammonium persulphate (APS) (Biorad), 0.1% sodium dodecyl sulphate (SDS) (BDH) and 0.06% N,N,N',N'-tetram-

ethylethylenediamine (TEMED) (Biorad). In gradient gels, glycerol (Fisons) was added to a final concentration of 0.08% in the 15% acrylamide gel mixture. The solutions were degassed by vacuum prior to the addition of SDS, APS and TEMED. After gels were poured, water saturated butan-2-ol was layered on top until the gel had polymerized, the butan-2-ol was then rinsed off with distilled water, the remaining space filled with distilled water, the gel plates sealed with cling-film and the gel left to overnight. This procedure was routinely followed to ensure as reproducible polymerization as possible between gels. A stacking gel, 5.5% acrylamide, 0.14% bis, 0.138M tris pH 6.8, 0.1% SDS, 0.8% APS and 0.06% TEMED was poured and allowed to polymerize for 30 minutes at room temperature.

10 µl sample were loaded per track of the mini gels, and up to 20µl per track on the large gels. Minigels were run at 50 volts, constant voltage through the stacking gel, and 150 volts through the running gel. Large gels were run at 20mA constant current through the stacking gel, and at 40mA through the running gel. In both cases a 0.025M tris base, 0.192 M glycine and 10% SDS running buffer system was used.

Pre stained protein molecular weight (14-200 Kd) markers (high range Gibco BRL) boiled in sample buffer were used as standards.

Table 4 Molecular Mass Markers

Protein	Reported Molecular Weight	Apparent Molecular Weight
Lysozyme	14,300	15,100
β-Lactoglobulin	18,400	17,850
Carbonic anhydrase	29,000	28,250
Ovalbumin	43,000	41,800
Bovine serum albumin	68,000	67,450
Phosphorylase B	97,400	103,150
Myosin (H-chain)	200,000	204,900

The coupling of the blue chromophore to the polypeptides of the molecular weight markers can affect their electrophoretic behaviour in SDS gels relative to the unstained protein. The above table shows the altered molecular weight of a typical batch of BRL prestained molecular weight markers.

The gel was either stained in Coomassie blue (0.05% Coomassie brilliant blue R (Sigma), 50% methanol, 10% acetic acid) and destained in 30% Methanol, 10% acetic acid and stored in 7% acetic acid, or processed for immunoblotting.

B. Protein Transfer to Nitrocellulose Membrane: Immunoblotting

i. Electroblothing

Two systems were used for protein blotting. For mini gels a Biorad Mini Trans-Blot electrophoretic transfer cell and an LKB 2197 power pack was used. The gel was rinsed briefly in transfer buffer (0.025 M tris base, 0.192M glycine, 20% methanol pH 8.3), placed onto prewet 3MM filter paper, covered by a piece of prewet nitrocellulose (0.45 μ m pore size, Schleicher and Schuell) cut to the exact size of the gel and finally a piece of 3MM filter paper. This was clamped in the gel holder cassette, placed in the electro-transfer cell and blotted for 1 hour at 100 volts constant voltage. For large gels an E-C Apparatus Corporation electroblot transblotter and power pack was used. Blotting was carried out as above at 750 mA for 2.5 hours.

ii. Post-blot Procedure

The blot sandwich was disassembled, and the SDS gel stained in Coomassie blue to ensure that protein transfer was successful. The nitrocellulose sheet was blocked overnight in 3% BSA, 0.1% NaN₃ in PBS, to reduce nonspecific binding by saturating free binding sites. The molecular weight markers were cut from the blot, rinsed in PBS and air dried. The rest of the blot was rinsed briefly in 0.3% BSA in PBS, placed on a sheet of parafilm, incubated with 0.50 μ g/ml of anti-transgelin in 0.3% BSA/PBS for 2.5 hours in a humid chamber, rinsed in 6 changes of 0.3% BSA/PBS for 30 minutes, and then incubated for 2.5 hours on a shielded shaker with 5 x 10⁶ counts of rabbit anti mouse Fab₂¹²⁵I in 0.3% BSA/PBS. The blot was washed with PBS until the counts dropped to 50 counts per

second, blotted on 3MM paper to remove excess moisture, baked in an 80°C oven until dry, then placed against Kodak XAR-5 film in a Genetic Research cassette containing X-Ograph intensifying screens, at -70°C. Exposure was usually overnight, the film was developed in an Agfa automatic developer, and the exposure time, temperature (-20°C, -70°C, or room temperature), film (XAR 5 or X Omat) and the presence or absence of intensifying screens (Dupont Cronex) varied to give the highest resolution of bands. Gels were photographed with an Olympus OM 1N camera and Macrolens, using Kodak Technical Pan film rated at 80 asa, which was developed in HC110, used at dilution D, for 4 minutes at 20°C to give a contrast index of 1.25. The photographs were printed on Kodak resin coated paper, grades 1-5 depending on the contrast requirement.

Alternatively, after the first antibody, the filter was incubated in rabbit anti mouse IgG conjugated to horseradish peroxidase in 0.3% BSA/PBS for 2.5 hours, rinsed in 6 changes of PBS, then developed by incubation in 0.05% 4-chloronaphthol (Sigma), 16.6% cold methanol, 0.015% hydrogen peroxide in PBS, for 15 minutes at room temperature. The filter was rinsed in PBS, air dried, and photographed.

Occasionally, prior to blocking the nitrocellulose was stained with amido black as follows, to visualize the total protein that had been transferred. The nitrocellulose filter was incubated for 2-3 minutes at room temperature in a solution of 0.1% amido black, 45% methanol, 2% acetic acid, removed and washed in 45% methanol, 2% acetic acid for 5 minutes and then dried without further rinsing.

C. Iodination

Rabbit anti mouse Fab₂ was iodinated by the chloramine T method. Chloramine T is a strong oxidizing agent and acts to convert iodide to iodine which is then free to bind to tyrosine groups in the protein (169). 100µg of antibody was conjugated to 200µCi of sodium iodide (IMS 30, Amersham), using chloramine T (Sigma) at a final concentration of 20µg/ml in a total volume of 1ml and after 90 seconds the reaction was stopped by the addition of sodium metabisulphate (Sigma) to a final concentration of 333µg/ml. The reaction mixture was made up to a volume of 1 ml with PBS, loaded onto a pre-

equilibrated Sephadex G25 column (P10 prepacked column, Pharmacia) and eluted with about 12 mls of PBS. 1ml fractions were collected, and 5 μ l samples of these fractions counted on a Nuclear Enterprises NE160 gamma counter. The fractions containing the antibody peak (which usually eluted at tubes 4-6) were pooled and stored at 4°C

Table 5 Iodination of Rabbit anti-Mouse Fab₂

Fraction number	Counts/min/ml
1	9.6 x 10 ³
2	2.7 x 10 ⁴
3	2.3 x 10 ⁴
4	4.9 x 10 ⁷
5	2.0 x 10 ⁸
6	9.6 x 10 ⁷
7	2.7 x 10 ⁷
8	1.0 x 10 ⁷
9	2.1 x 10 ⁷
10	1.9 x 10 ⁵
11	1.3 x 10 ⁴
12	9.6 x 10 ³

Samples 4-6 were pooled and used in experiments. 43 μ l / 20mls PBS were used to give 5 x 10⁶ counts.

D. ⁴⁵Calcium Binding Assay

To measure calcium binding activity, 5 μ g purified transgelin (see section 4B), and 5 μ g parvalbumin (Sigma), a known calcium binding protein (291) were transferred to a nitrocellulose filter, section , washed in 3 changes of buffer (60mM KCl, 5mM MgCl₂, 10mM Imidazole HCl pH 6.8) for an hour, incubated in 100 mls of the same buffer with 100 μ Ci ⁴⁵calcium chloride (CES 3, Amersham) for 10 minutes, rinsed in distilled water

for 2.5 minutes, then 50% ethanol for 2.5 minutes, blotted dry on filter paper, dried in an 80°C oven and exposed overnight to XAR film. (291)

E.pI Determination

i. NEPHGE gels

Protein C4, purified from REFs on an antibody affinity column (section 4A) was boiled in Laemmli sample buffer and frozen at -70°C until required. 10µl of this SDS sample was equilibrated for NEPHGE gels by the addition of 5µl of undiluted Nonidet P40 (NP40, BDH), 10 µl of isoelectric focussing (IEF) sample buffer (9.5M Urea Ultrapure, Schwartz Mann, 2% SDS, 2% pH 3.5-10 Ampholines, Pharmacia, 5% 2-mercaptoethanol) and solid urea, to maintain a final concentration of 9.5M. An excess of NP40 was added so that SDS bound to the protein would be replaced by NP40, a non-ionic detergent, thus preventing streaking of the protein in the first dimension. The sample was loaded onto a 3mm i.d. x 13 cm glass tube, containing a 12cm polymerized NEPHGE gel (9.2M Urea, 2% NP 40, 4% acrylamide (of stock 28.38% Acrylamide, 1.62% bis), 2% pH 3.5-10 ampholines, APS, TEMED), overlaid with 20µl of overlay solution (6M urea, 1.1% ampholines), and electrophoresed with 0.02M sodium hydroxide in the lower chamber (cathode solution) and 0.01M phosphoric acid in the upper chamber (anode solution), and run at 400 volts, constant volts for 4 hours. The pI generated by these conditions was measured by running a blank gel in parallel, cutting this into 1 cm pieces, equilibrating them in 4 mls of distilled water for 1 hour and measuring pH. The gels were gently extruded from the glass tubes, equilibrated in 2x Laemmli sample buffer (section 3Aia) for 2 hours, then run in the second dimension on a 17 x 13 cm 12% SDS gel, with a 1 inch stacking gel. Tube gels were sealed on top of the second dimension gel using 1% agarose in running buffer, with bromophenol blue as a marker (245). To visualize the protein, the gel was stained with silver nitrate as follows.

ii. Silver Staining

The gel was prefixed in 50% methanol, 10% acetic acid for 30 minutes, 5% methanol, 7% acetic acid for 45 minutes, fixed for 30 minutes in 2% glutaraldehyde, then rinsed in several changes of distilled water for 2 hours, and soaked in 5 µg/ml DTT for 30

minutes. This solution was poured off, and without rinsing, 0.1% silver nitrate added for 30 minutes. The gel was rinsed rapidly in distilled water, then in 2 changes of developer (0.3% sodium carbonate, 0.0185% glutaraldehyde) and soaked in fresh developer until the desired level of staining was attained. The staining reaction was stopped by the addition of 0.015M citric acid to the developer. After 10 minutes, this was discarded and the gel washed in several changes of distilled water for 30 minutes prior to being photographed as above. The gel was stored in 0.03% Na₂CO₃, to prevent bleaching of the stain.

F. In Vivo Metabolic Labelling/ Immunoprecipitation

i. In Vivo Metabolic Labelling

5 x 10⁴ REFs/ml were plated overnight in E4 + 10% FCS in 75mm² Falcon tissue culture flasks, and incubated overnight at 37°C, 5% CO₂ (as section 1Ci), rinsed in E4 - methionine (supplied by ICRF) + 2% FCS, and incubated in this depleted medium for 30 minutes. This was replaced by 3.3 mls of fresh E4 - methionine + 2% FCS, containing 0.5 mCi/ml of [³⁵S] methionine (SJ 204, Amersham), the cells incubated for 4 hours, rinsed in E4 - methionine for 30 seconds and either chased in complete E4 + 10% FCS for 48 or 72 hours in adherent or suspension culture (see section 1ci,iii), or trypsinized immediately, washed in E4 + 10% FCS, then PBS.

ii. Immunoprecipitation

Pellets of cells from either chased or unchased experiments were lysed by re-suspending them in 100µl of lysis buffer (10mM Tris pH 7.5, 2mM EDTA, 1mM PMSF, 0.1mM leupeptin, 1µg/ml soy bean trypsin inhibitor (SBTI) and 0.5% Triton X-100) for 10 minutes at 0°C. The total lysate was centrifuged at 10,000g for 10 minutes at 4°C, the detergent soluble supernatant carefully removed from the Triton insoluble pellet and precleared by the addition of 10µg of normal mouse IgG [prepared by DEAE fractionation (see section 2Diii)] for 1 hour at 4°C, followed by 50µg of affinity purified rabbit anti mouse IgG (Cappel) for 2 hours at 4°C and centrifuged at 10,000 g for 10 minutes at 4°C.

The precleared supernatants were incubated with preformed immune complexes at 4°C overnight. Immune complexes had previously been formed by incubating 10 µg of monoclonal anti C4 or a mouse monoclonal IgG anti β tubulin (Amersham) with 50 µg of affinity purified rabbit anti mouse IgG in a total volume of 45µl lysis buffer overnight at 4°C. The crosslinked immune complexes were washed twice by centrifugation at 10,000 g for 10 minutes in 100 µl lysis buffer at 4°C and then added to the precleared supernatants in a total volume of 45µl and gently resuspended.

The samples were centrifuged as above and the pellets washed by 4 centrifugation steps: 2 x in 100µl lysis buffer, 1 x in 100µl lysis buffer + 0.5M NaCl, and finally in 100 µl lysis buffer alone. Pellets were solubilized in Laemmli sample buffer (see section 3Aia) and approx. 3.5×10^4 counts/slot loaded onto 12-15% gradient gels (see section 3Aii).

The gels were fluorographed with Amplify (Amersham International), dried on a Biorad slab gel drier and exposed to X-Omat AR film. The position of the C4 doublet was determined by running a parallel track of affinity purified C4 from REFs (section 4A), the gel lightly stained with Coomassie Blue, dried and the bands marked with radioactive ink before exposure on film.

To determine the ratio of transgelin : lower band, the precipitated C4 immune complex was blotted (3Ai) and labelled with anti-protein C4/rabbit anti-mouse Fab₂^{125I} and the ratio determined by excising the bands from the nitrocellulose and counting the strips in a gamma counter.

4. Protein Purification

A. Purification of Protein C4 on Antibody Affinity Columns

For preliminary investigations to establish the basic characteristics of the protein, transgelin purified from a monoclonal antibody affinity column was used.

10 mg of monoclonal anti-C4 were coupled to Affigel 10 (Biorad) by the following method. 20 mls of resuspended Affi-gel was washed in 3 bed volumes (30 mls) of isopropanol, then 3 bed volumes of distilled water, at 4°C. The moist gel cake was transferred to a 30 ml universal, 10 mgs of antibody in 0.1M MOPS, pH7, added, and

incubated for 4 hours with gentle shaking at 4°C. Unreacted sites were blocked with by the addition of 1ml of 1M ethanolamine HCl, pH 8, for 1 hour at 4°C. The gel was spun at 180 g for 5 minutes, (the supernatant retained and antibody concentration remaining uncoupled determined by OD₂₈₀ measurement), transferred to a column and washed with 3 bed volumes of 0.1M MOPS pH7, then 50mM Tris HCl pH7. Typically, coupling efficiency was 90%.

1 x 10⁶ REFs were harvested, washed in PBS, lysed for 25 minutes in 15 ml 50 mM Tris HCl pH 7, 0.5% Triton X-100, 1µg/ml SBTI, 1mM PMSF, 100µM leupeptin, 2mM EDTA, 5mM NaN₃ at 4°C, and then spun at 20, 000g for 30 minutes to remove insoluble material. Supernatants were passed over the column at a flow rate of 5ml/hour, the column washed down to background with 50mM Tris HCl pH 7, 0.5% Triton X-100, and the bound fraction eluted with 50 mM diethylamine pH 11.5, immediately neutralized with 1M Tris pH 7, dialyzed into 50 mM Tris pH 7 and concentrated to 1 ml with Aquacide (Mr 70 kD, Calbiochem). The purity of the sample was determined by SDS-PAGE immunoblotting, followed by amido black staining to visualize total protein, or anti-protein C4 antibody and rabbit anti-mouse Ig peroxidase (Dakopatts).

B. Purification of Transgelin from Smooth Muscle

To bypass the unavoidable denaturation caused by elution of protein from an antibody affinity column I developed the purification protocol outlined below.

Sheep aortas were collected from the abattoir of Ziff Meats Ltd, stripped of fat, connective tissue, cut into strips and placed directly into liquid nitrogen, within 1 hour of slaughter. The aortas were transported and stored in liquid nitrogen.

Pieces of aorta were removed from liquid nitrogen, weighed (usually 10-12g for each preparation), placed in polythene bags, and rapidly fractured into small pieces (less than 1 cm²) with a precooled aluminium block. These pieces were then thawed/homogenized in 5 volumes of wash buffer (60mM KCl, 4mM MgCl₂, 1mM NaN₃, 0.5 mM EGTA, 10mM Imidazole pH7, 5mM DTT, 0.5% CHAPS, 0.1mM EDTA, 0.2mM PMSF) using a Waring blender on maximum setting for 3 x 10 seconds. This, and all subsequent steps were carried out at 4°C and in the presence of leupeptin, chymostatin

and pepstatin, all at 2 µg/ml. After 1 hour at 4°C, with frequent mixing, the detergent insoluble material was removed by centrifugation at 23,000 g for 5 minutes, the pellet discarded and the supernatants, after filtration over glass wool, dialysed overnight against 2L of high salt/low pH buffer (50mM Na Acetate, 4 mM MgCl₂, 0.5 mM EGTA, 0.5M KCl, pH 4.8) and clarified by centrifugation at 48,000 g for 2 hours. The supernatant was dialysed overnight into chromatofocusing start buffer (25mM ethanolamine HCl pH 9.6). A chromatofocussing column (Pharmacia polybuffer exchanger, packed in a Pharmacia C16 x 70 column) was pre-equilibrated overnight using 10-15 bed volumes of start buffer, which had been degassed and cooled. To avoid exposing samples to extremes of pH, 5 mls of elution buffer (Pharmacia Polybuffer 96, diluted 1:10 with precooled degassed distilled water, made to pH 7 with 0.5M HCl) were run onto the column, then the sample applied and eluted with elution buffer at a flow rate of 12-15 ml/hr to maximise the resolution of the column. 3ml fractions were collected using an LKB Multirac S111 fraction collector, connected to an LKB Uvicord S11 spectrophotometer and monitored by an LKB 2210 chart recorder. The column was regenerated by washing with 1.0M NaCl in distilled water and re-equilibration with start buffer.

The peak containing transgelin, as determined by SDS-PAGE analysis (see section), was dialysed overnight against 20mM K Phosphate buffer (KH₂PO₄/K₂HPO₄ pH 6.8) and as a final purification step was applied to a small (3cm³ bed volume) hydroxylapatite column, (Biogel HT, Biorad), in a 10 x 1 cm Econocolumn, which had been pre-equilibrated with 20 mM K Phosphate buffer, and eluted with 100mM K Phosphate. 1 ml fractions were collected (as above), and the protein peak dialysed into 100mM K Phosphate overnight, and the concentration of transgelin determined by reading OD₂₈₀. If necessary, the concentration was increased by filtration with Millipore ultrafree microcentrifuge tubes, which were centrifuged at 6,500 g for up to 30 minutes. Transgelin was stored at 4°C and used within 3-4 days, or aliquoted, and stored at -70°C. Samples were taken at each stage of the purification and analysed by SDS-PAGE.

The purity of the samples were tested by SDS-PAGE, immunoblotting and probing with anti-protein C4 antibody. A single peak was found by reverse phase chromatography (Dr J Hsuan and Dr N Totty, Ludwig Institute).

C. Purification of Actin

The method of Pardee and Spudich (339), with minor modifications was used to purify actin from rabbit skeletal muscle (410). Hind leg and dorsal muscles of a rabbit were removed, cooled and washed in ice water, and ground in a meat mincer previously chilled to 4°C. The minced muscle was weighed, extracted in 3 volumes of phosphate KCl buffer (150 mM KH_2PO_4 , 300 mM KCl, to pH 6.5 with 10M KOH, at 4°C) for 10 minutes with stirring, and this was then diluted by addition of 12 volumes of distilled water (4°C) with vigorous stirring for 5 minutes. The suspension was filtered through sterile cheesecloth, the residue resuspended in 5 volumes of 0.4% NaHCO_3 , 0.1mM CaCl_2 , stirred for 30 minutes at 4°C, filtered as above, the residue resuspended in 1 volume of 10 mM NaHCO_3 , 10 mM Na_2CO_3 , 0.1 mM CaCl_2 and stirred for 10 minutes at 4°C. This suspension was diluted with 10 volumes of distilled water, stirred for 3 minutes and filtered. The residue was resuspended in 2 volumes of acetone, precooled to 4°C and stirred for 30 minutes at room temperature, and filtered. This extraction was repeated at least 5 times (until the filtrate appeared to be free of fat). The dehydrated acetone powder was allowed to air dry overnight, and then stored in 5g aliquots at -20°C, with silica gel (Sigma) as a desiccant.

To obtain actin, the acetone powder was extracted at 4°C for 2 hours by stirring with 20 ml G buffer (2mM Tris-HCl, pH 8.0 at 25°C, 0.2mM Na_2ATP , 0.5mM 2-mercaptoethanol, 0.2mM CaCl_2 , 0.005% NaN_3) per g acetone powder. The supernatant was separated from the hydrated acetone powder by centrifugation at 40,000 g for 30 minutes, and decanted by pipetting. To polymerise the actin in this supernatant into filaments, the KCl concentration of the supernatant was brought to 50mM, MgCl_2 to 2mM, and ATP to 1mM, and incubated at 4°C for 2 hours to allow for assembly. To remove tropomyosin from the actin preparation, solid KCl was added slowly with stirring to a final concentration of 0.6M, and the solution stirred gently for 30 minutes at 4°C. The polymerised actin filaments were collected by centrifugation at 100,000 g for 16 hours at 4°C, the supernatant discarded, the pellets homogenised gently in 100 ml of fresh buffer and centrifuged at 150,000 g for 2 hours. After discarding the supernatant, the pellets were softened by allowing them to stand in 200µl of G buffer at 4°C overnight. They were

transferred to a dounce homogenizer and gently homogenized by hand with a teflon coated rod. To completely depolymerize the actin, the homogenate was dialysed against G buffer for 3 days at 4°C, changing the buffer every 24 hours, to ensure that non hydrolysed ATP was available. F actin oligomers and polymers were removed by centrifugation at 150,000 g for 2 hours. Dialysed actin was further purified by gel filtration on Sephadex G150 (Pharmacia), packed in a Pharmacia C100 x 2.6 column, which had been equilibrated with cold, degassed G buffer. 20 mg G actin in 8 mls of G buffer was applied to the column and eluted with G buffer at a flow rate of 10 mls/hour. 10 ml fractions were collected using an LKB fraction collector (see section 2 D ii). Peaks were analysed by SDS PAGE, and those fractions containing actin alone were pooled, concentrated against PEG 6000 (Sigma) and dialysed against fresh G buffer overnight. Immediately prior to use the dialysed actin was clarified by centrifugation in a Beckman airfuge at 148,000 g for 100 minutes at 4°C, the pellets discarded, and the actin concentration of the supernatant measured by reading OD₂₈₀. Actin was kept in G buffer for 5-6 days only, after which time it was cycled by polymerization/depolymerization. Actin to be stored as an F actin pellet was polymerised by the addition of KCl, Na₂ATP and MgCl₂ as above, NaN₃ added to 0.2%, the filaments pelleted by centrifugation as above, the supernatant discarded and replaced with fresh buffer and the pellets stored in filled, sealed tubes at 4°C.

5. Sedimentation Assay

A pelleting assay was used to examine the interaction of transgelin with F actin. A test sample of total volume 100µl was prepared by mixing water, 10 x Buffer P, transgelin and finally actin to a concentration of 9.3 µM in an airfuge tube (Beckman polyallomer centrifuge tube 5 x 70 mm), incubating for 1 hour at 25°C, and centrifuging in a Beckman Airfuge at 148,000 g for 1 hour at 4°C. The outside edges of the tubes were marked before centrifugation, to enable supernatants to be withdrawn carefully, with hand drawn Pasteur pipettes, without disturbing the pellets. Supernatants and pellets were suspended in Laemmli sample buffer (3Aia) to give total sample volumes of 150 µl, electrophoresed on 12% SDS PAGE (3Bi) and stained for 1 hour with Coomassie Blue, destained for 1 hour and stored in 7% acetic acid. The gels were scanned with a Hoefer GS300 densitometer

linked to a Macintosh SE 30 using a GS370 electrophoresis data reduction system to determine the amounts of protein in each band. The densitometer had previously been calibrated by scanning SDS PAGE gels containing known amounts of transgelin. To determine the binding constant K_d , and saturation point, varying amounts of transgelin, from 0.6 to 12 μ M were added to the reaction mixtures. The concentrations of bound versus free protein were plotted and a concentration curve obtained, and the binding constant was taken to be 1/free transgelin concentration at the point where the ratio of bound: free transgelin is half its maximal value (492).

6. Functional Assays

A. Viscosity Assays

i. Low Shear Viscometry

A falling ball viscometric assay system was used to assay the effect of transgelin on actin viscosity (352).

100 μ l micropipettes (Corning, 12.7 cm long, 1mm i.d.) were cut in half lengthways, and marked along their length at 1 cm intervals. A test sample of 144 μ l volume was prepared in a microfuge tube by mixing distilled water, 10 x buffer P (final concentration 2mM $MgCl_2$, 1mM ATP, 1mM EGTA, 12mM K phosphate pH 6.8) transgelin and finally G actin monomer, prepared as above, to give a final concentration of 9.3 μ M. The water, actin and buffer were all warmed to room temperature before use. Addition of the actin initiated the reaction, so the sample was quickly drawn into the previously prepared micropipettes by capillary action. The capillary tube was sealed at one end with Clay Adams Seal Ease, held at an angle of 50° to the horizontal and incubated at room temperature for 30 minutes.

A stainless steel ball (0.6mm in diameter, kindly provided by Dr S McIver) was placed by hand on the meniscus of the sample and pushed gently through the meniscus with a fine platinum wire to initiate its fall. The velocity of the ball was measured by recording the time required for the ball to pass 1 cm intervals, beginning 0.5 cm below the meniscus (352).

ii Effect of Transgelin Concentration

To assay the effect of transgelin concentration on actin viscosity, viscometry experiments were carried out in the absence and presence of varying amounts of transgelin, up to a concentration of 4.4 μM

iii Time Course of Gelation

To determine the time course of transgelin induced actin gelation, the capillary tubes were set up as above, and the velocity of the ball bearing and therefore apparent viscosity measured at various time points after the addition of actin to the reaction mixture, from 15 seconds to 30 minutes.

iv Effect of Ionic Strength on Transgelin Induced Gelation

To determine the effect of ionic strength on actin viscosity, KCl was added to the reaction mixture, up to a concentration of 100mM. Experiments were also carried out in the presence of Na tripolyphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$) (Sigma) from 0-4 μM , to study the effects of positive ions on the transgelin actin interaction (310).

v. Interaction of Transgelin with G Actin

Transgelin was incubated with G actin monomers for 30 minutes at 4°C, prior to the addition of buffer P to the reaction mixture, to determine whether transgelin interacted with G actin. Viscosity readings were taken at various times after the initiation of the polymerization reaction.

vi Effect of Transgelin on Actin Filament Annealing

To determine whether transgelin would anneal short actin filaments, a reaction mixture was set up in a microfuge tube, excluding transgelin, and the actin allowed to polymerize for 30 minutes at room temperature. The filaments were then broken by pipetting 5 times with a Finn pipette, transgelin added to 2.4 μM , and this mixture drawn into a micropipette and allowed to stand for 30 minutes at room temperature (439,311). The velocity of the ball bearing, and therefore the apparent viscosity, was measured as above.

vii Effect of pH on Transgelin : Actin Interactions

The pH of the reaction mixture was altered from pH 6-9 by altering the ratio of the acidic and basic components of the K phosphate buffer, and the effect of this on the system examined.

viii Effect of Calcium on Transgelin : Actin Interactions

To see whether Ca^{2+} was involved in the control of transgelin induced actin gelation, CaCl_2 up to 0.2 mM or EGTA, which chelates calcium, up to 2 mM.

B. Pyrene Actin Fluorescence Assays

i. Preparation of Pyrene Labelled Actin

Pyrene labelled actin was prepared as follows. G actin (prepared as in section) before the gel filtration step was dialysed against 0.2mM CaCl_2 , 0.5mM ATP, 2mM Tris HCl pH 7.5 to remove 2 - mercaptoethanol, and diluted to a final concentration of 40 μM in the same buffer. 5 mM pyrene iodoacetamide (Molecular Probes Inc), freshly dissolved in DMSO (Sigma) was added dropwise to a final concentration of 45 μM to a rapidly stirring actin solution. KCl and MgCl_2 were added to 100mM and 2 mM respectively and the actin allowed to polymerise for 16 hours, in the dark at room temperature. The pyrene labelled F actin was collected by centrifugation at 100, 000 g for 3 hours, resuspended in G Buffer, depolymerised by dialysis against G buffer + 1mM NaN_3 and centrifuged to remove insoluble material at 400, 000 g for 2 hours. The pyrene labelled G actin supernatant was chromatographed on a Sephadex G150 2.5 x 50 cm column run at 10 ml /hour. Concentrated fractions of pyrene labelled actin monomers as determined by SDS PAGE analysis were pooled, the optical density at 344 nm and 290 nm measured and the total actin and pyrene: actin ratio calculated. For immediate use pyrene labelled G actin was stored on ice with 0.5mM ATP added. 0.5 ml aliquots of pyrene actin at 1mg/ml were frozen in liquid nitrogen and stored at -85°C . Before use, they were thawed quickly and centrifuged at 148, 000 g for 40 minutes.(52,232)

ii. Nucleation assay

To determine whether transgelin affected the initial nucleation step of actin polymerisation, G actin ($5\mu\text{M}$) of which 20% was pyrene labelled was induced to polymerise in the presence or absence of transgelin ($1.29\mu\text{M}$) by the addition of 10x Buffer P. Distilled water, actin, buffer and transgelin were all pre-equilibrated to 25°C . The sample, total volume $300\mu\text{l}$, was mixed in a cuvette, so that the changes in fluorescence were measured continuously as soon as possible after the initiation of polymerisation. Measurements were made in a MPF 3L Perkin Elmer spectrophotometer with excitation and emission wavelengths set at 366 and 408 nm respectively and slit widths at 10 nm.

iii. Filament Elongation Assay

To determine whether transgelin enhanced or retarded the elongation of existing F actin filaments, G actin ($5\mu\text{M}$) was induced to polymerise by the addition of Buffer P in the presence or absence of transgelin ($1.29\mu\text{M}$). After an incubation period of 30 minutes at 25°C , a further $5\mu\text{M}$ G actin, of which 20% was pyrene labelled were added to the preformed filaments, and changes in fluorescence intensity measured as above.(78)

C. Light Scattering Assay

A light scattering assay was used to further investigate the changes in crosslinking of actin filaments in the presence of transgelin under different ionic conditions. $9.3\mu\text{M}$ G actin $\pm 2.4\mu\text{M}$ transgelin were polymerised in Buffer P $\pm 100\text{ mM KCl}$ in a total volume of 0.3 ml. All solutions were previously equilibrated to 25°C . The changes in 90° light scattering were measured continuously as soon as possible after the initiation of polymerisation in a spectrofluorimeter (MPF 3L Perkin Elmer) with excitation and emission wavelengths both set at 360 nm and slit widths at 2 nm.(78,228)

D. Electron Microscopy

This work was done in collaboration with my supervisor Dr D Lawson. $0.5\mu\text{M}$ G actin was incubated for 1 minute or 10 minutes $\pm 2.4\mu\text{M}$ transgelin in Buffer P and then fixed by the addition of 5% glutaraldehyde in Buffer P to a final concentration of 0.5%.

Carbon formvar grids were glow discharged at 1 A for 2 minutes and pretreated for 1 minute with cytochrome c (0.2 mgs/ml in 0.1% isoamyl alcohol, Sigma). Excess liquid was removed by blotting with damp filter paper and immediately 18 μ l aliquots of actin filaments were allowed to adsorb onto the grids for 10 - 15 minutes at room temperature. Excess liquid was removed as above, the grid treated again with cytochrome c, rinsed with 1% methanol in molecular biology grade distilled water (Sigma) and then negative stained with 15 μ l 1% aqueous uranyl acetate + 1% methanol for 90 seconds. Excess stain was removed and the grid allowed to air dry before viewing in an electron microscope (100 CX 11; Jeol) operating at 80 kV.(212,311)

7. Linear Sucrose Density Gradients

Sucrose density gradients were carried out to determine whether transgelin occurred as a monomer, dimer, trimer or tetramer in physiological and low (12mM) ionic conditions.

5% and 20% sucrose solutions were made up in 12mM or 100mM K Phosphate buffer + 2mM $MgCl_2$, 1mM EGTA. 10 ml 5-20% linear gradients were poured using a Biorad Gradient former in 14 ml polyallomer centrifuge tubes (Kontron), and were left at 4°C overnight. Lyophilised protein standards were made up to a concentration of 0.5 mg/ml in the same buffers, and were incubated at 4°C for 2 hours to solubilise. Standards included soy bean trypsin inhibitor (SBTI) Mr 21kDa, ovalbumin Mr 43kDa, BSA Mr 68kDa and transferrin Mr 80kDa (Sigma). To remove any aggregates the solutions were centrifuged at 10,000 g for 10 minutes at 4°C and filtered with syringe filters (Sartorius 0.22 μ m pore size). 0.5 ml of each protein standard was carefully layered on top of separate gradients, taking care to avoid bubble formation, the interface between the protein and the sucrose gradient gently stirred, and allowed to stand at 4°C for 15 minutes. 0.5 mls of transgelin at 0.5 mgs/ml in 12mM or 100mM K phosphate buffer was loaded onto a separate gradient. The gradients were centrifuged at 150,000g for 43 hours at 4°C, after an initial very slow acceleration (10 seconds) of the rotor, the rotor was allowed to reach full speed, and deceleration at the end of the run was slow with the brake turned off. The tubes were then clamped into a retort stand, punctured at the base with a syringe needle, and 1 ml fractions collected in microfuge tubes. A fresh needle was used for each tube to maintain

drop size. The protein concentration of the fractions was monitored by reading OD_{280} . Fractions corresponding to the peak readings at 280nm were saved, concentrated by methanol/chloroform extraction (3Aicii) and analysed by SDS PAGE (3Aii). (287)

RESULTS

In the results section, unless otherwise stated, the anti protein C4 antibody used, and designated anti-protein C4 antibody, is a monoclonal antibody. The term protein C4 is used to designate the doublet comprised of both higher and lower molecular mass isoforms, while the higher molecular mass isoform (formerly C4^h) is called transgelin, and the lower molecular mass isoform, C4^l.

1 Distribution of Protein C4 in Normal Mesenchymal Cells

Immunofluorescence studies on REFs, fixed in methanol and incubated with either monoclonal or polyclonal anti-protein C4 antibody, showed, at least at the level of the light microscope, intense, uniform staining along the whole length of actin stress fibre bundles (Fig 1a and b). Identical staining patterns were seen when anti-protein C4 and the F-actin specific probe fluorescein phalloidin were compared in double immunofluorescence experiments (Fig 2a). Stress fibres labelled with phalloidin but not anti-protein C4 antibody were not seen (Fig 2a and b).

Neither the cortical actin cytoskeleton, cell processes, nor the non linear meshwork of actin filaments, found more deeply inside the cells were stained by anti-protein C4 antibody (Fig 1a and b).

In cells treated with cytochalasin D which fragments actin filaments (without affecting cell shape) the stress fibres, labelled with fluorescein phalloidin were shown to collapse into dense foci (Fig 2d). This redistribution of actin was paralleled by protein C4, as demonstrated by the coincident labelling of actin and protein C4 (Fig 2c and d). However, the disruption of the intermediate filament network by colchicine, shown by anti-vimentin labelling (Fig 2f), left the distribution of protein C4 undisturbed (Fig 2e).

2 Distribution of Protein C4 in Various Cell Types

In cells such as transformed mesenchymal cells (Fig 3b and c) or epithelial cells (Fig 3a), where stress fibres are reduced in number or absent, protein C4 antibody staining had a more diffused distribution throughout the cytoplasm, with some staining seen on the vestigial stress fibres found in the former.

3 Biochemical Expression of Protein C4

SDS PAGE coupled with immunoblot analysis of total protein from normal rat fibroblasts showed that both the monoclonal and polyclonal anti C4 antibody recognized a closely spaced doublet of polypeptides, transgelin and C4^l (Fig 4a and 6a). These polypeptides were usually present in equal amounts, although in some tissues and species, for example sheep aorta, transgelin was found in excess of C4^l (Fig 8b). The molecular weight of transgelin was shown to be 21 kDa, whilst that of the lower band was 0.5-1 kDa less. These values were calculated using BRL prestained molecular weight markers as standards for a plot of % mobility against log₁₀ molecular weight. The molecular weights of transgelin and C4^l were calculated (Fig 6i).

The molecular weight of both transgelin and C4^l were unaltered by increased concentrations of reducing agents (Fig 4b and c) or the presence of protease inhibitors (Fig 4d).

4 Detergent Solubility of Protein C4

By immunofluorescence with anti-protein C4 antibody, protein C4 was removed from REFs by extraction for 5 minutes at 0°C with 0.5% of the non-ionic detergent Triton X-100 (Fig 5a).

This finding was confirmed by SDS PAGE, followed by immunoblot analysis using anti C4 antibody of detergent insoluble pellets and detergent soluble supernatants of REFs, which had been extracted for 5 minutes at 4°C in 0.5% Triton X-100, centrifuged for 1 minute at 10,000g and carefully separated, prior to processing for SDS PAGE. Protein C4 was present only in the soluble extract (Fig 5c); all detectable protein C4 had been removed from the insoluble cytoskeleton fraction (Fig 5b).

Figure 1 Localisation of protein C4 on stress fibres in fibroblasts

REFs, permeabilised with methanol were labelled with anti-protein C4 antibody and anti mouse IgG/rhodamine (a). Phase contrast image of the same cell (b) shows that protein C4 staining coincides with stress fibres.



Figure 2 Immunofluorescence localization of protein C4 in REFs

Double immunofluorescence labelling with anti-protein C4 (a) and fluorescein phalloidin (b) in control REFs, and anti-protein C4 (c) and fluorescein phalloidin in cytochalasin D treated REFs shows that protein C4 distribution parallels that of actin. Anti-protein C4 labelling is undisturbed in colchicine treated cells (e) which induces the collapse of intermediate filaments (labelled with rabbit anti-vimentin) around the nucleus (f).

Bar : 20 μ m

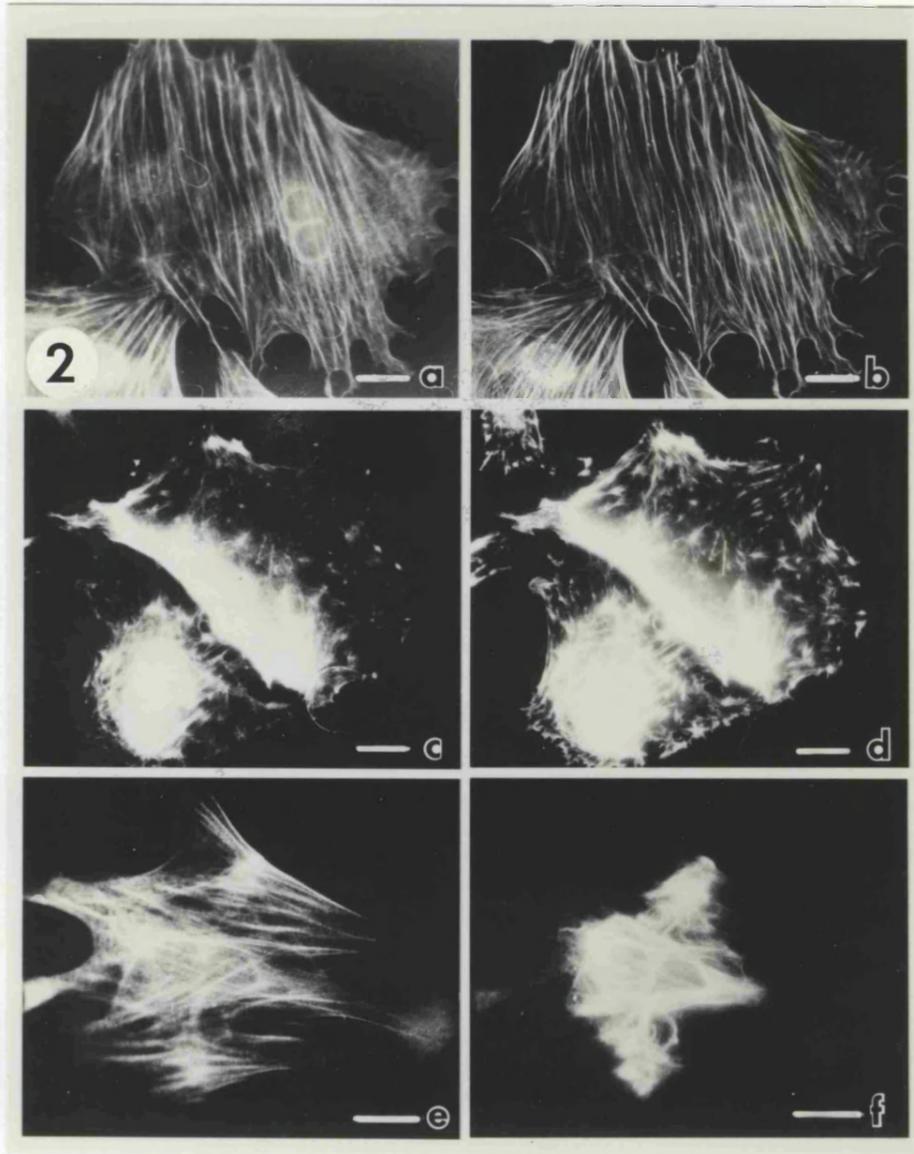


Figure 3 Immunofluorescence localization of protein C4 in other cell types.

Diffuse non-linear cytoplasmic protein C4 labelling is seen in epithelial cells (Detroit 98)

(a). In SV40 3T3 transformed cells, some anti-protein C4 labelling is seen along vestigial stress fibres (b) Phase contrast image of the same cell (c).

Bar : 10 μ m

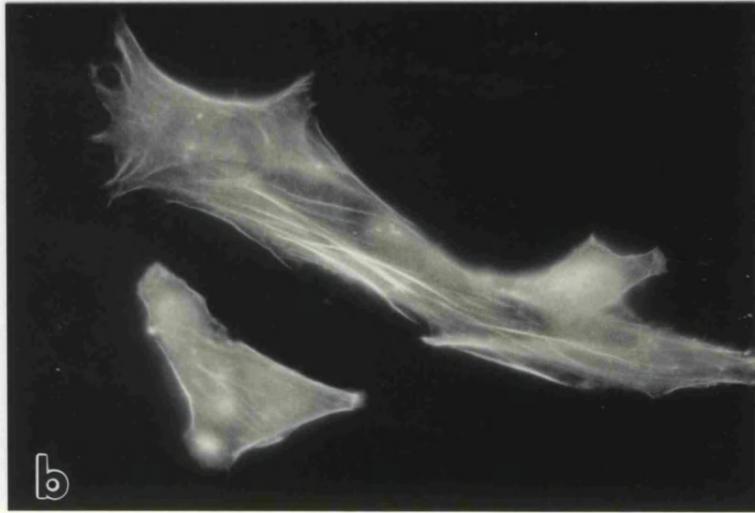
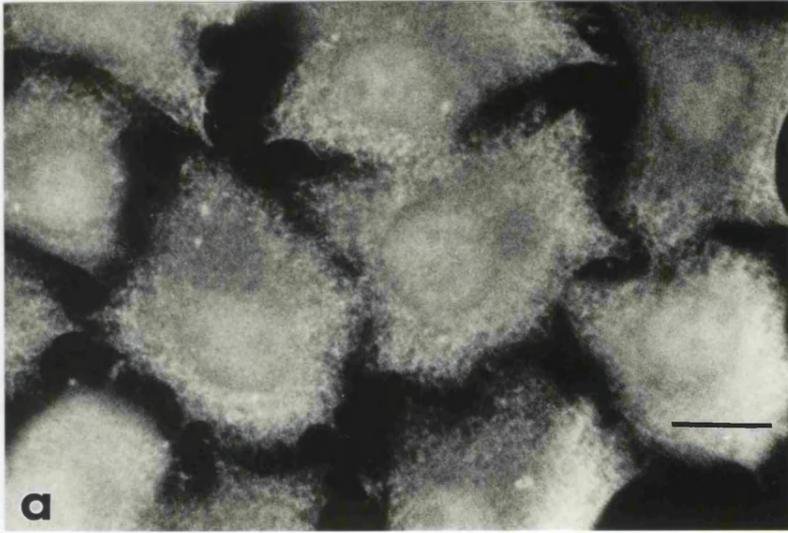


Figure 4 Immunoblot analysis of protein C4 polypeptide doublet

REFs were solubilised in standard sample buffer (a) and in sample buffer + 10% 2mercaptoethanol (b) and + 100mM DTT (c).

Mouse 3T3 fibroblasts were solubilised in the presence of a spectrum of protease inhibitors (d) (5-15% gradient SDS PAGE gel)

Immunoblotted tracks were incubated with anti protein C4 antibody. The closely spaced polypeptide doublet is unaffected by the presence of excess reducing agents or protease inhibitors.

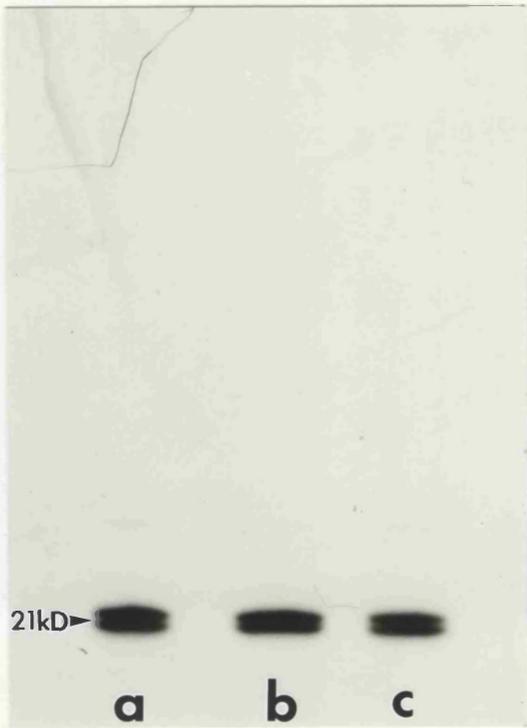


Figure 5 Detergent solubility of protein C4

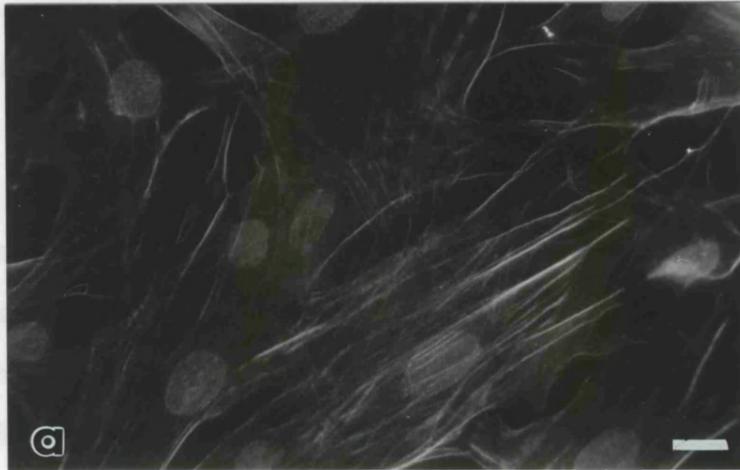
Anti protein C4 labelling is lost in REFs extracted for 5 minutes at 0°C with 0.5% Triton X-100 in wash buffer (a).

REFs extracted as above were centrifuged for 1 minute at 10,000 g and (b) pellets and (c) supernatants analysed by SDS immunoblotting. Labelling with anti-C4 antibody shows that protein C4 is present only in the detergent soluble supernatant (c).

Bar : 10µm

5 Distribution of Protein C4 in Vertebrate Tissues

When present, transgelin was co-expressed with C4^I. Transgelin has not yet been found without the simultaneous expression of C4^I, although it is in examples same tissues for example, smooth muscle (rat per Fig 5b, sheep aorta Fig 7b). It is absent not only from neurones, erythrocytes and muscle (Fig 6a and 7a), and epithelial cells (Fig 6c) but also present in the cytoplasm of fibroblasts (Fig 6d), keratinocytes (Fig 6e), brain (Fig 6f), skeletal muscle (Fig 7c), neurons and immunocytes (Fig 7d). Transgelin is associated with actin filaments (Fig 8d).



6 Comparison of Protein C4 with Proteins of Similar Mass

To exclude the possibility that anti-protein C4 antibody recognized a previously identified protein, samples of proteins with similar mass to protein C4 were analysed by SDS PAGE immunoblotting. Anti-protein C4 antibody did not recognize cofilin (Fig 9g), ADF (Fig 9f) or N-ras p21 (Fig 9e).



7 Conservation of Protein C4

Protein C4 have been found in vertebrates so far examined - frog (Fig 6c), chicken (Fig 6e), fish (Fig 6d), mouse (Fig 7h), sheep (Fig 7b) and human (Fig 7a). When the same tissue was examined, the molecular mass was constant while that of C4^I varied from 17.5 kDa in chickens to 19.5 kDa in humans. While immunoblot of yeast was negative, when identical immunoblots were performed with monoclonal anti-protein C4 antibody, strong staining was seen at 21kDa (Fig 10a). Identical labelling pattern (Fig 10b).

5 Distribution of Protein C4 in Various Tissues

When present, transgelin was co-expressed with C4^l. Transgelin has not yet been found without the concomitant expression of C4^l, although it is in excess in some tissues for example, smooth muscle (rat gut Fig 6b, sheep aorta Fig 7b). It is absent not only from neurones, erythrocytes, lymphocytes, skeletal muscle (Fig 7c), brain (Fig 6g and 7a), and epithelial cells (Fig 6h), but also from heart, where a separate band of 79kDa was present (Fig 6f).

C4^l was found in all cell and tissue types of rat so far examined (REFs Fig 6a, brain Fig 6g and 7a, smooth muscle Fig 6b and 7a, heart Fig 6f) apart from skeletal muscle (Fig 7c), neurones and erythrocytes.

Immunoblot analysis of sheep aorta (Fig 8b) and isolated smooth muscle actin thin filaments (Fig 8d), prepared as described (285), showed that protein C4 is associated with actin filaments (Fig 8d).

6 Comparison of Protein C4 with Proteins of Similar Mass

To exclude the possibility that anti-protein C4 antibody recognized a previously identified proteins, samples of proteins with similar mass to protein C4 were analysed by SDS PAGE immunoblotting. Anti-protein C4 antibody did not recognize cofilin (Fig 9g), ADF (Fig 9f) nor N-ras p21 (Fig 9b and d).

7 Conservation of Protein C4

Protein C4 have been found in all vertebrate species so far examined -frog (Fig 6e), chicken (Fig 6c), fish (Fig 6d), rat (Fig 6b and 7b), mouse (Fig 8h), sheep (Fig 8b) and human (Fig 6h). When the same tissue (smooth muscle) from different vertebrate species was examined, the molecular mass of transgelin remained constant while that of C4^l varied from 17.5 kDa in chickens to 19.7 kDa in rats.

While immunoblots of yeast were unlabelled by the monoclonal anti-protein C4 antibody, when identical immunoblots were incubated with polyclonal anti-C4 antibody, strong staining was seen at 21kDa (Fig 10b). Control tracks of purified transgelin showed an identical labelling pattern (Fig 10d).

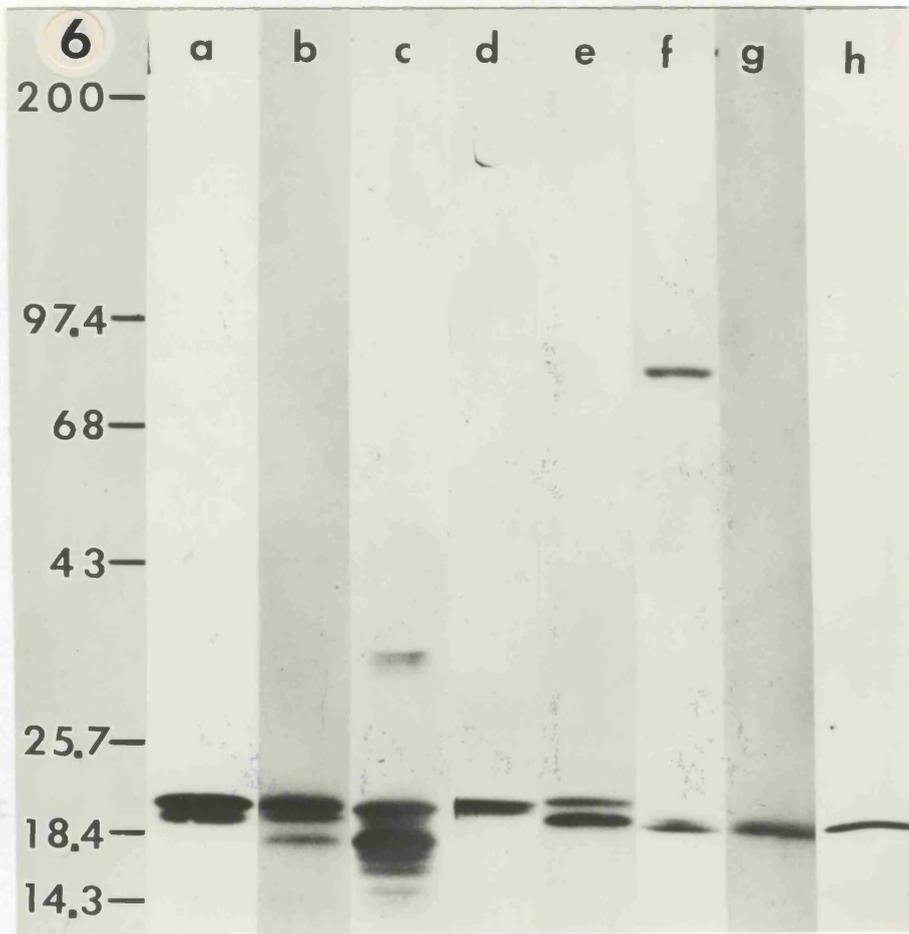
Figure 6 Immunoblot analysis of protein C4 in different cell and tissue types

Immunoblot tracks of total solubilised tissue/cells were labelled with anti-protein C4 antibody and anti-mouseIg Fab₂ ¹²⁵I(a-h).

Protein C4 is present as a doublet of 21kDa in normal mesenchymal cells such as REFs(a).

The relative molecular mass of C4^l from different tissues of the same species remains constant (a) rat fibroblasts (b) rat smooth muscle (gut) (f) rat heart and (g) rat brain. Some variation in the quantity and relative molecular mass of C4^l is found when the same tissue type (Smooth muscle) from different species is compared (b) rat gut (c) chicken gizzard (d) fish gut (e) Xenopus gut. Transgelin is absent in (f) heart, where an additional band at 79kDa is seen (g)brain and in (h) Detroit 98 human epithelial cell line.

A graph (overleaf) was used to calculate the relative molecular mass of protein C4.



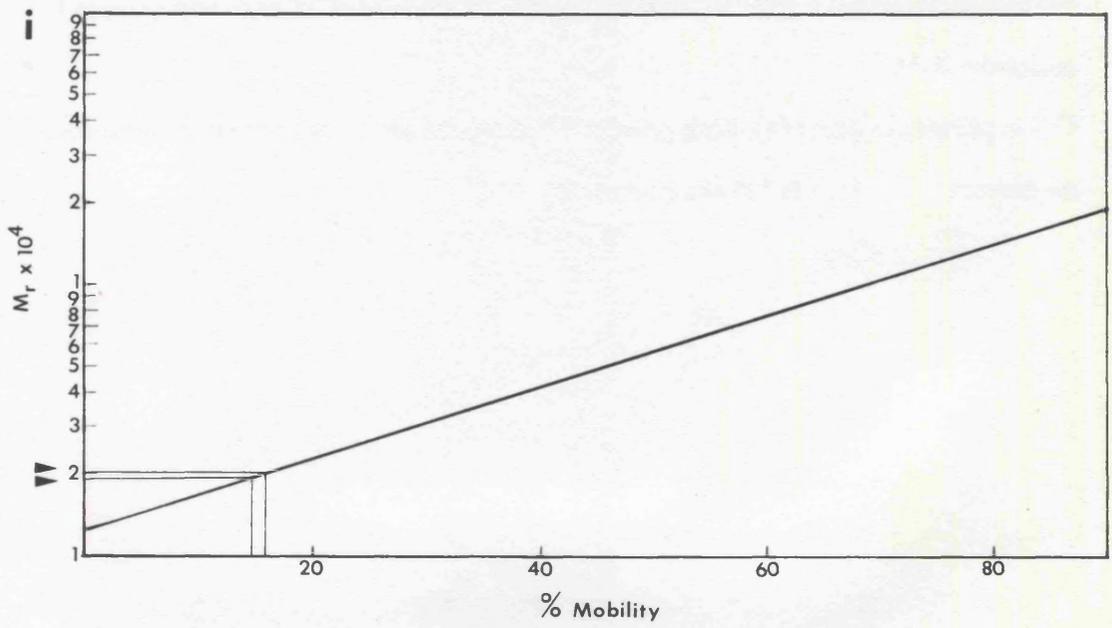


Figure 7 Immunoblot analysis of Protein C4 in rat tissues

Immunoblot tracks of total solubilized rat tissues were labelled with anti-protein C4 antibody (a-c).

C4I is present in brain (a), both protein C4 isoforms are present in smooth muscle (b) and no protein C4 is detected in skeletal muscle (c).

21kD ▶



a b c

Figure 8 Immunoblot analysis of protein C4 in sheep aorta and actin thin filaments

Total solubilized sheep aorta (Coomassie blue stained track, a) immunoblotted and labelled with anti-protein C4 antibody (b) shows that protein C4 is present in this tissue, with transgelin in excess of C4¹. Protein C4 (d) remains associated with aorta thin filament preparations, to which several other actin associated proteins are bound(285) (Coomassie blue stained track, c). Removal of these actin associated proteins from actin thin filaments (by increased ionic strength and removal of ethandiol) (Coomassie blue stained track, e) is paralleled by the absence of anti-protein C4 label (f). As a control total solubilized mouse 3T3 fibroblasts (g) were immunoblotted and labelled as above (h) A protein C4 doublet is clearly seen.

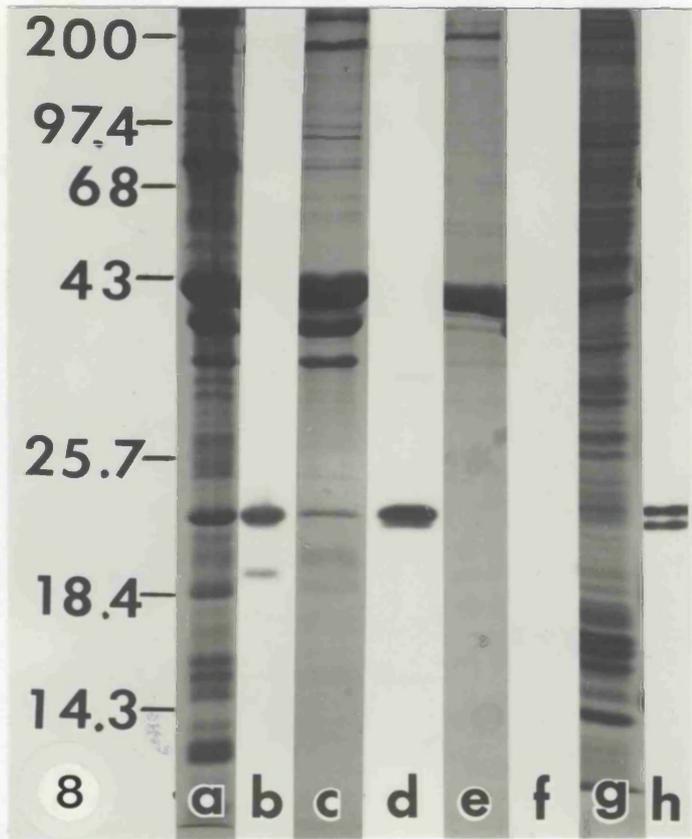


Figure 9 Comparison of protein C4 with other proteins of Mr 21 kDa

Coomassie blue stained tracks of prestained molecular mass markers (a) 5 μ g of N-ras p21 (b) and total REFs (c). When 5 μ g N-ras p21 was immunoblotted and labelled with anti-protein C4 antibody no staining was seen (d) Anti protein C4 antibody recognises a polypeptide doublet at Mr 21kDa in an immunoblot of total REFs (e).

Purified ADF (f), cofilin (g) and total REFs were immunoblotted and labelled with anti-protein C4. Anti-protein C4 labelling is only seen in REFs

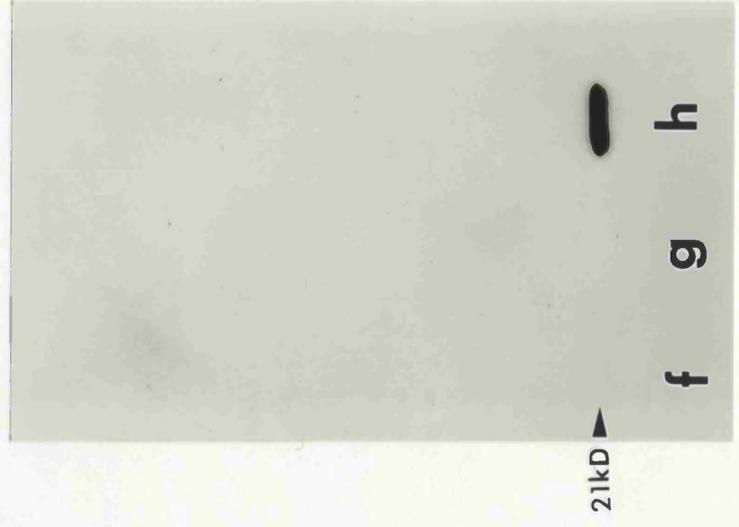
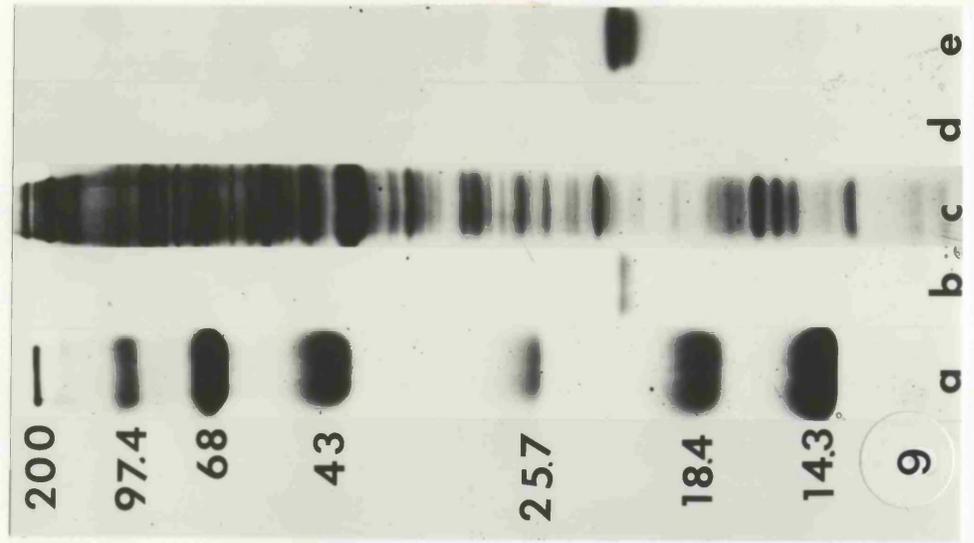


Figure 10 Transgelin in fission yeast *S. pombe*

- (a) Coomassie blue stained track of total solubilised *S. pombe*.
- (b) Immunoblot of total *S. pombe*. labelled with polyclonal anti-transgelin antibody shows a single band running in parallel with purified transgelin [in tracks (c) and (d)].
- (c) Coomassie blue stained track of 5 μ g of purified transgelin.
- (d) Immunoblot of purified transgelin labelled as in track (b)

8 Effect of Oncogene Transformation on Protein C4

The higher molecular mass protein C4 isoform, transgelin, was never found when normal 3T3 fibroblasts which had been transformed with the DNA tumour virus, Simian virus 40 (SV40) were examined by SDS PAGE/immunoblotting (Fig 11a). In contrast, transgelin was always found in normal, untransformed 3T3 cells (Fig 11b). An identical result was obtained when Rat-1 cells, transformed with either normal RNA virus - Rous sarcoma virus or

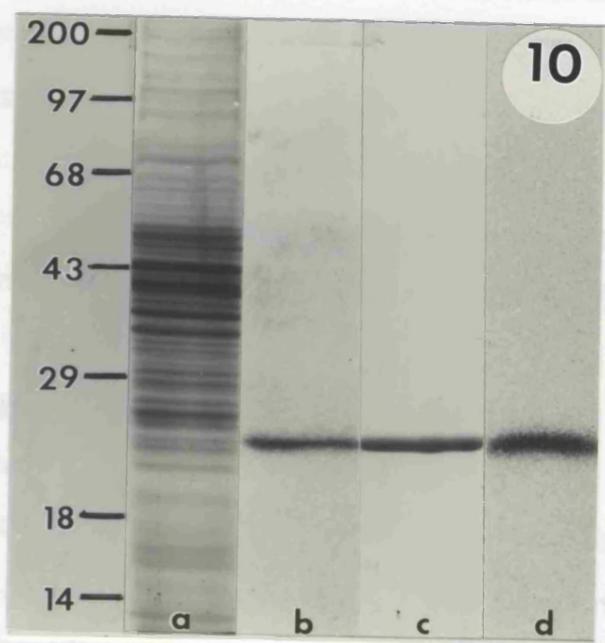
SV40 (Fig 11c) or untransformed Rat-1 cells (Fig 11d) were examined by immunoblot analysis. To contrast, cells transformed with either SV 40 (Fig 11a) or RSV (Fig 11c) were examined by immunoblot analysis (Fig 11b and d).

9 Effect of Cell Shape

i Suspension Culture
The loss of transgelin expression could be attributed by culturing normal cells in suspension. Cells assumed a rounded morphology (Fig 11e) and showed a progressive loss of transgelin over a 72 hour period by which time no transgelin was detectable by immunoblot analysis (Fig 11e). Identical results were found with 3T3 cells, a human fibroblast line, and REFs (not shown). When cells which had been in suspension culture for 72 hours (Fig 11e) were allowed to reattach and spread, transgelin expression was not detectable after 8 hours (Fig 11f), but attained its normal level of expression after 24 hours in adherent culture (Fig 11g). The levels of C4¹ expression was apparently unaffected by suspension culture (Fig 11g-i).

ii Effect of Metabolic Inhibitors on Transgelin Re-expression after Suspension Culture

After 72 hours in suspension, some cells were reseeded in adherent culture for 24 hours but in the presence of either actinomycin D or cycloheximide. Both of these metabolic inhibitors blocked re-expression of transgelin as seen by immunoblotting (Fig



8 Effect of Oncogenic Transformation on Protein C4

The higher molecular mass protein C4 isoform, transgelin, was never found when normal 3T3 fibroblasts which had been transformed with the DNA tumour virus, Simian virus 40 (SV40) were examined by SDS PAGE/immunoblotting (Fig 11a). In contrast, transgelin was always found in normal, untransformed 3T3 cells (Fig 11b). An identical result was obtained when Rat-1 cells, transformed with either normal RNA virus - Rous sarcoma virus or a temperature sensitive RSV mutant (LA 29) were examined by immunoblot analysis (Fig 11c). Transgelin was present in untransformed Rat 1 cells (Fig 11d).

In contrast, C4^l expression was unaltered in immunoblots of either SV 40 (Fig 11a) or RSV (Fig 11c) transformed cells and their normal counterparts (Fig 11b and d).

9 Effect of Cell Shape Change on Protein C4 Expression

i Suspension Culture

The loss of transgelin in oncogenically transformed fibroblasts could be mimicked by culturing normal, fibroblastic cells in suspension. 3T3 cells, prevented from attaching to the substrate and spreading normally by covering the petri dish with silicon grease, assumed a rounded morphology (Fig 11o) and showed a progressive loss of transgelin over a 72 hour period by which time no transgelin was detectable by immunoblot analysis (Fig 11e). Identical results were found with Butlers, a human fibroblast line, and REFs (not shown). When cells which had been in suspension culture for 72 hours (Fig 11g) were allowed to reattach and spread, transgelin expression was not detectable after 8 hours (Fig 11h), but attained its normal level of expression after 24 hours in adherent culture (Fig 11i). The levels of C4^l expression was apparently unaffected by suspension culture (Fig 11g-i).

ii Effect of Metabolic Inhibitors on Transgelin Re-expression after Suspension Culture

After 72 hours in suspension, some cells were returned to adherent culture for 24 hours but in the presence of either actinomycin D or cycloheximide. Both of these metabolic inhibitors blocked re-expression of transgelin as seen by immunoblotting (Fig

11j andk), while in control experiments, normal re-expression of transgelin occurred within 24 hours (Fig 11i). Cells incubated in the presence of metabolic inhibitors appeared to reattach and assume a normal well spread morphology (Fig 11m).

In parallel experiments, cells cultured in suspension for 72 hours were plated onto coverslips, and allowed to reattach and spread for various times in the presence or absence of actinomycin D and cycloheximide. They were then processed for immunofluorescence and labelled with monoclonal anti-protein C4 antibody at 0, 8, and 24 hours after removal from suspension culture. The numbers of actin stress fibre bundles, and the degree of protein C4 staining, were recorded, to look for any correlation between the expression of transgelin and the presence of these stress fibre bundles.

At t_0 , the cells were rounded and intense cytoplasmic staining with anti-protein C4 was seen (Fig 11o), but there were no stress fibre bundles visible. After 8 hours of respreading in the absence of inhibitors, only 13% of the cells had visible stress fibres and anti-protein C4 staining was faint and mainly cytoplasmic (Fig 11l). However, after 24 hours, when the transgelin band had reappeared (Fig 11i), 72% of cells had large numbers of actin stress fibre bundles traversing the cytoplasm and the anti-protein C4 staining was seen along their entire length (Fig 11n).

In contrast, of the cells which had respread for 24 hours in the presence of either actinomycin D or cycloheximide, only 10% and 13% respectively had stress fibres and these were vestigial compared with control cells (Fig 11m).

Table 6

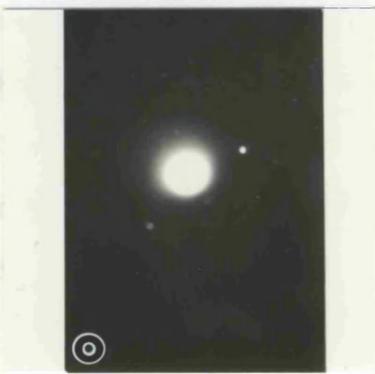
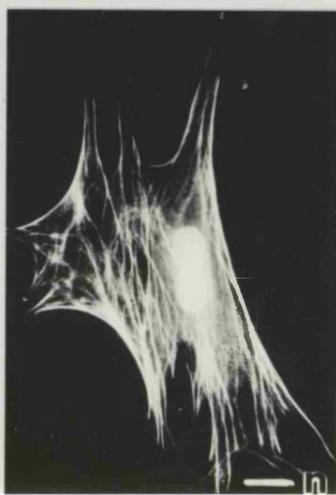
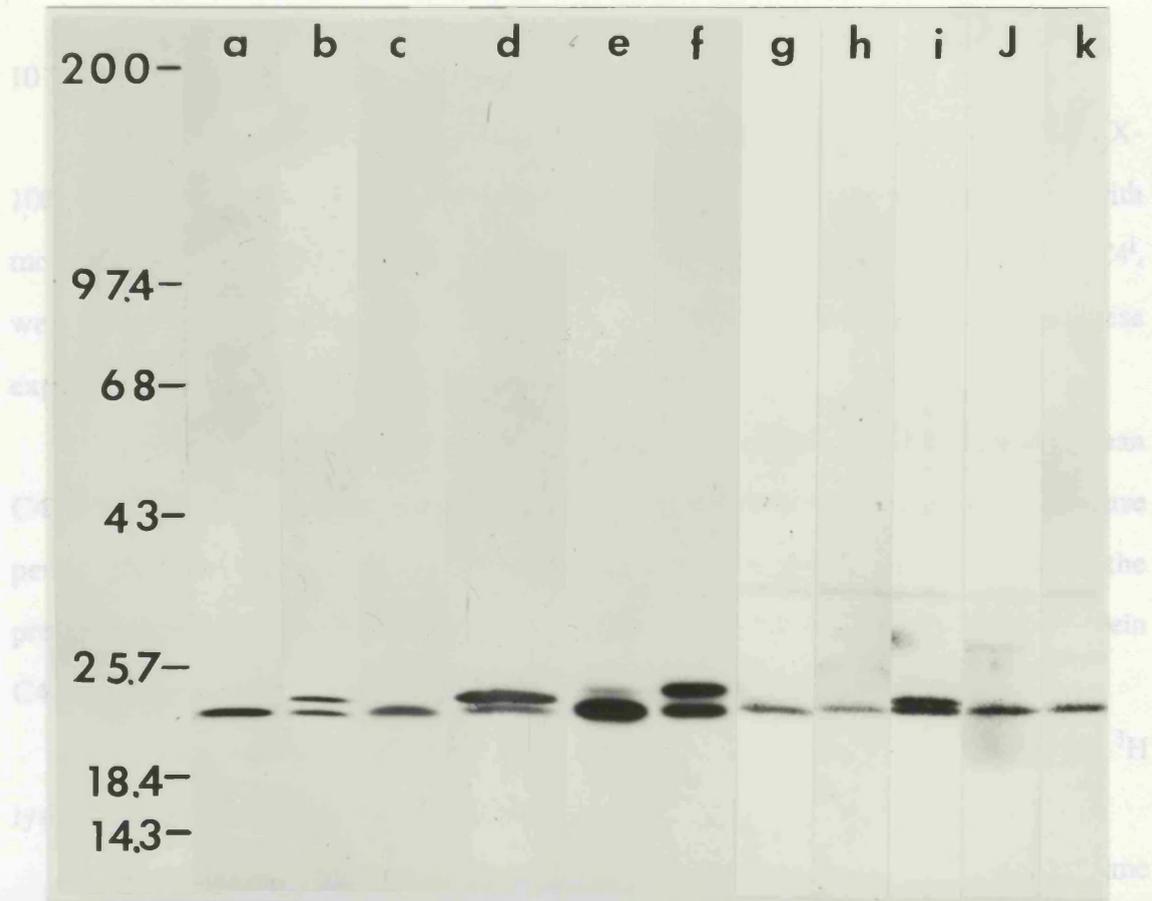
Time: hours after respreading	% Cells with actin stress fibre bundles		
	Control	+Actinomycin D	+Cycloheximide
0	0	0	0
8	13	nk	nk
24	72	10	13

nk - not known

Figure 11 Differential expression of protein C4 in transformed and shaped changed cells

Total solubilized cells were immunoblotted and labelled with anti-protein C4 antibody. Transgelin is absent in SV40 transformed 3T3 cells (a) and RSV transformed mutant LA29 cells (c), but is present in their normal counterparts 3T3 cells (b) and rat 1 fibroblasts (d). This oncogenically induced down regulation of transgelin is mimicked by culturing 3T3 cells in suspension culture for 72 hours when only traces of transgelin are left, but C4^l remains (e) whereas both protein C4 isoforms are expressed in equal amounts in 3T3 cells in adherent culture (f). Re-expression of transgelin could not be detected when cells that had been in suspension culture for 72 hours (g) were allowed to reattach and spread for 8 hours (h). After 24 hours in adherent culture, transgelin expression had reached normal levels (i). This re-expression of transgelin is blocked by either actinomycin D (j) or cycloheximide (k).

Immunofluorescence labelling of cells in parallel show that after 72 hours in suspension culture cells are rounded and show intense cytoplasmic anti-protein C4 staining and no stress fibre are visible(o). After 8 hours reattachment in adherent culture few stress fibre bundles are present (l) but after 24 hours in adherent culture many stress fibres have reformed and are labelled with anti-protein C4(n). Incubation of suspension cultured cells in either cycloheximide or actinomycin D during the 24 hour adherent culture period inhibited the formation of stress fibres in most cells, and anti-protein C4 labelling remains diffuse (m).



nitrocellulose and incubated with anti-protein C4 antibody followed by goat anti-mouse Ig peroxidase (Fig 13 a). These as a doublet of 21 kDa, with experimental conditions (Fig with amino black to virus polypeptides present (Fig 13)

ence of both transgenin and C4 greater than the latter in these to nitrocellulose but incubated that these were the only two

10 In Vivo Metabolic Labelling/Immunoprecipitation

In vivo metabolic labelling of REFs with [³⁵S] methionine, followed by Triton X-100 extraction of these cells, and immunoprecipitation of the detergent soluble fraction with monoclonal anti-protein C4 antibody showed that two bands at 21 kD, transgelin and C4^l, were co-precipitated (Fig 12b). A band at 18 kD was occasionally seen in these experiments.

After a 4 hour labelling period transgelin had incorporated more radioactivity than C4^l (Fig 12b). No reduction nor change in this ratio was seen after a 48 hour chase period (Fig 12c). A ratio of 1 : 2.5 - 1 : 4.33 C4^l : transgelin was found when the precipitated immunocomplexes were transferred to nitrocellulose, probed with anti-protein C4 / anti-mouse Fab₂ ¹²⁵I, the bands cut out and counted on a gamma counter.

Identical results were obtained when the cells were labelled with ³H leucine and ³H lysine (not shown).

Control experiments using an anti β tubulin monoclonal antibody, of the same class as the monoclonal anti-protein C4 antibody used (IgG₁), showed that a single band was precipitated at 50 kDa with no bands visible at 21 kDa (Fig 12d).

11 Protein C4 Purification

i Affinity Column

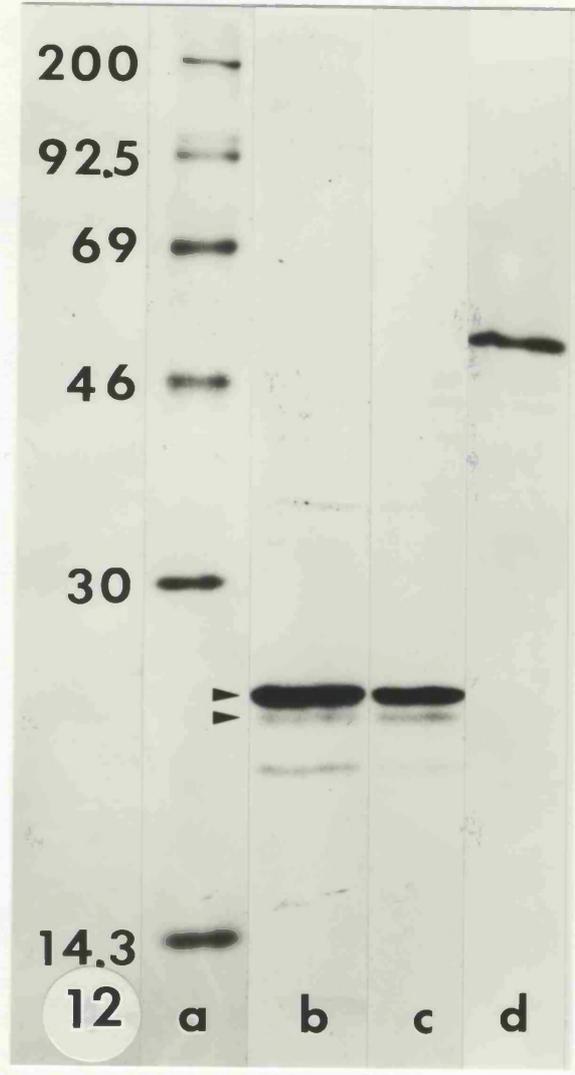
By affinity column purification on monoclonal anti-protein C4 antibody conjugated to Affigel 1.58μg (SD ± 0.4) purified protein C4 was obtained from 1 x 10⁶ REFs. To assay the purity of the preparation the eluted peak was run on SDS PAGE, transferred to nitrocellulose and incubated with anti-protein C4 antibody followed by goat anti-mouse Ig peroxidase (Fig 13 a). These experiments showed the presence of both transgelin and C4^l as a doublet of 21 kDa, with the recovery of the former greater than the latter in these experimental conditions (Fig 13a). Parallel tracks transferred to nitrocellulose but incubated with amido black to visualise total protein showed that these were the only two polypeptides present (Fig 13b).

Figure 12 In vivo metabolic labelling/Immunoprecipitation

- (a) Autoradiograph of ^{14}C labelled molecular weight markers
- (b) REFs after 4 hour pulse with [^{35}S] methionine were lysed and immunoprecipitated with anti-protein C4 antibody. Two bands at Mr 21 kDa, transgelin and C4^l are present (arrowheads). Transgelin contains approximately 2-4 times as much radioactivity as C4^l.
- (c) Immunoprecipitate of protein C4 from REFs after 4 hour [^{35}S] methionine pulse and 48 hour chase. Both bands are present and there is no apparent change in the amount of radioactivity incorporated into the 2 bands during this 48 hour chase period.
- (d) A control immunoprecipitate of β tubulin from REFs has a single band at 50 kDa.

Table 7 Affinity Column Purification of Protein C4

Fraction number	Protein concentration (mg/ml)	OD ₂₈₀	Protein C4 (μg/ml)
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Fractions 3-14 were pooled, giving a total yield was 1.50 μg protein C4, from 1×10^8 cells, as determined by a Bradford protein assay kit.

Table 7 Affinity Column Purification of Protein C4

II Two Dimensional Gel Analysis of Affinity Purified Protein C4

NEPHGE gel Fraction number gradient of 4 - 9.3 OD₂₈₀ experimental conditions described in methods (0.5 mls)

Fraction number	OD ₂₈₀	Protein
1	0.003	
2	0.00	
3	0.00	
4 (end)	0.03	
5	0.20	
6	0.35	
7	0.33	Actin
8	0.33	
9	0.16	
10	0.15	
11	0.14	C4
12	0.18	Transgelin
13	0.14	
14	0.13	
15	0.09	
16	0.09	
17	0.09	
18	0.09	
19	0.16	
20	0.09	
Base line	0.004	

NEPHGE coupled with SDS PAGE showed that transgelin and C4 were focused in the Fractions 5-14 were pooled, giving a total yield was 1.58µg protein C4, from 1 x 10⁶ cells, as determined by a Biorad protein assay kit. 8 and 7 µl, respectively (Fig 13c). Actin, run in a parallel control gel was shown to migrate 3.2 cm from the anodal end of the gel, giving a pI of 5.

ii Two Dimensional Gel Analysis of Affinity Purified Protein C4

NEPHGE gels generated a pH gradient of 4 - 9.3 in the experimental conditions described in methods section.

Table 8 NEPHGE Gel

Section; cms from anode	pH measured	
(+ve end)		
1	4.19	
2	4.4	
3	5.04	Actin
4	6.07	
5	6.87	
6	7.55	C4 ¹
7	8.10	Transgelin
8	8.58	
9	9.04	
10	9.01	
11	9.22	

NEPHGE coupled with SDS PAGE showed that transgelin and C4¹ were focused in this system. Measuring the position of the two polypeptides from the anodal end of the gel, it was determined that transgelin and C4¹ have 8 and 7 pI, respectively (Fig 13c). Actin, run in a parallel control gel was shown to migrate 3.2 cm from the anodal end of the gel, giving a pI of 5 .

Figure 13 Affinity column purification of protein C4

Protein C4 isolated from REFs was affinity column purified and analysed by SDS PAGE immunoblotting.

(a) Amido black staining of immunoblot of purified protein C4 shows that protein C4 is the only protein present in the sample.

(b) Anti-protein C4 label of (a)

(c) Silver stain of NEPHGE gel of purified protein C4 shows that transgelin and C4^l have pI of 8 and 7 respectively. (see table)

12 Purification of Transgelin from Sheep Aorta

A variety of purification protocols were tested before settling on the method described in *Methods*. A denaturation step with different purification protocols.

Sheep aorta was this tissue is a rich source of C4 is present in negligible amounts can be collected and stored. Steps were performed at further minimize degradation.

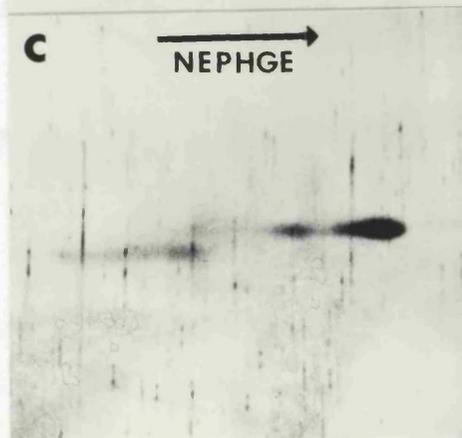
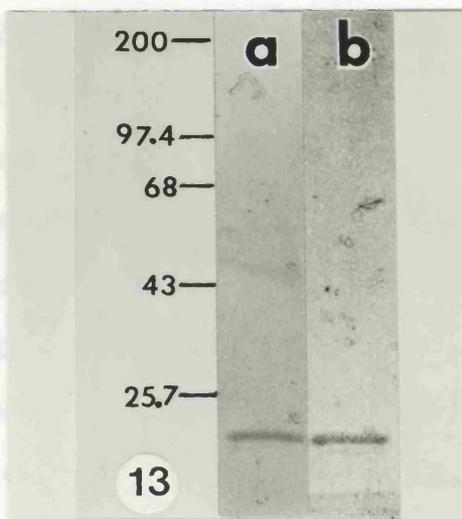


Table 9 Protease Inhibitors

Inhibitors	Concentration
Chymostatin	2 $\mu\text{g/ml}$
Leupeptin	Chymotrypsin, chymotrypsin-like serine proteases, most cysteine proteases
Pepstatin	Cathepsin D, pepsin, renin and metalloproteinases
PMSF	All serine proteases and cysteine proteases
EDTA	Metalloproteases and Ca^{2+} -dependent cysteine proteases

To isolate transgelin from aorta, I exploited our previous finding that it is highly detergent soluble, and homogenized frozen, fragmented pieces of aorta in 0.5% CHAPS, a

12 Purification of Transgelin from Sheep Aorta

A variety of purification protocols were tested before standardising on the method described in section. Although the affinity column was successful, the unavoidable denaturation that occurs when protein is eluted from an affinity column dictated that a different purification protocol was developed to bypass this problem.

Sheep aorta was used for the following reasons; (a) we have previously shown that this tissue is a rich source of transgelin (b) in this tissue the lower molecular mass isoform, C4^l, is present in negligible amounts (Fig 8b and d, Fig 14b) and (c) large amounts of aorta can be collected and stored with no apparent loss of protein C4 activity. All purification steps were performed at 4°C, and in the presence of the protease inhibitors listed below to further minimize degradation of the protein.

Table 9 Protease Inhibitors

Inhibitors	Protease targets	Concentration
Chymostatin	Chymotrypsin, chymotrypsin-like serine proteases, most cysteine proteases	2 µg/ml
Leupeptin	Chymotrypsin, chymotrypsin-like serine proteases, most cysteine proteases	2 µg/ml
Pepstatin	Cathepsin D, pepsin, renin and microbial aspartic proteases	2 µg/ml
PMSF	All serine proteases and cysteine proteases	0.2mM
EDTA	Metallo-proteases and Ca ²⁺ dependent cysteine proteases	0.1mM

To isolate transgelin from aorta, I exploited our previous finding that it is highly detergent soluble, and homogenized frozen, fragmented pieces of aorta in 0.5% CHAPS, a

zwitterionic detergent. Most of the transgelin was extracted from aorta during this initial homogenization/detergent extraction/centrifugation step. Transgelin remained in the detergent soluble supernatant (Fig 14c). While many detergent insoluble contaminating proteins were removed by centrifugation, much actin and tropomyosin remained in the supernatant (Fig 14c), and to remove this I used high salt, and a pH of 4.8 near to the pI of actin. Overnight dialysis of detergent soluble supernatants in these conditions followed by centrifugation, precipitated most actin and tropomyosin (Fig 14d), but left transgelin in the supernatant (Fig 14e). A protein of 68kDa remained in the supernatant at this stage (Fig 14e).

Chromatofocusing was used to further purify transgelin. A gradient of pH 9.6 - 7 was used, and transgelin eluted at a pH of 8.0. While negligible amounts of the 68 kDa protein was found in the peak containing transgelin, most of this contaminant was removed by this method (Fig 15).

Since the transgelin containing peak from the chromatofocusing column was present in a large volume of approximately 50 mls, and contained trace amounts of contaminating proteins I used an hydroxylapatite column as a final purification and concentration step. This also separated out polybuffer from the sample. The peak eluted from this column (Fig 16) was analysed by SDS PAGE and shown to contain a single band (Fig 16e). Immunoblotting, followed by staining with anti protein C4 antibody showed that this band (Fig 14g) migrated in the same position as transgelin present in control tracks of fibroblasts, which also contained C4¹, the lower molecular mass isoform (Fig 14h). No degradation was apparent by either Coomassie Blue staining or immunoblot analysis. Reverse phase chromatography showed the presence of a single peak (J Hsuan and N Totty) From data from protein sequencing, the estimated extinction coefficient for transgelin is 1.08 at OD₂₈₀.

From 10 g of aorta, 1mg of transgelin was obtained. Purified transgelin was stored on ice at 4°C, in the presence of leupeptin, chymostatin and leupeptin (2µg/ml each) and was used for functional studies within 4 days.

Figure 14 Purification of transgelin.

- (a) Coomassie blue stained track of total aorta
- (b) Immunoblot of total aorta labelled with anti-protein C4 antibody shows that transgelin is the major protein C4 isoform present in this tissue.
- (c) Coomassie blue stained track of detergent soluble supernatant after incubation of aorta in 0.5% CHAPS at 0°C for 30 minutes and centrifugation at 23,000 g for 5 minutes. Transgelin is present in this fraction.
- (d) Pellet following dialysis in 0.5M KCl at pH 4.8 and centrifugation at 48,000 g for 2 hours. Most of the contaminating actin is removed by this step, while little transgelin is lost in the pellet.
- (e) Supernatant after incubation in 0.5 M KCl at pH 4.8 and clarification as above. Transgelin is present in this fraction.
- (f) Coomassie blue stained track of purified transgelin following chromatofocussing and hydroxylapatite column chromatography.
- (g) Immunoblot of purified transgelin labelled with monoclonal anti-protein C4 antibody shows that transgelin is the only protein C4 isoform present. No degradation of transgelin is detectable.
- (h) Immunoblot of total REFs labelled as in (g) to show the presence of both protein C4 isoforms.

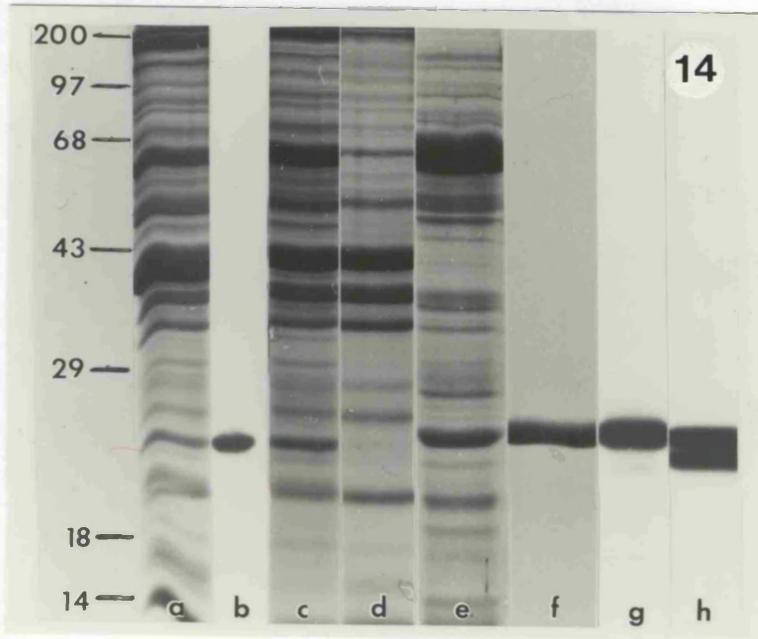
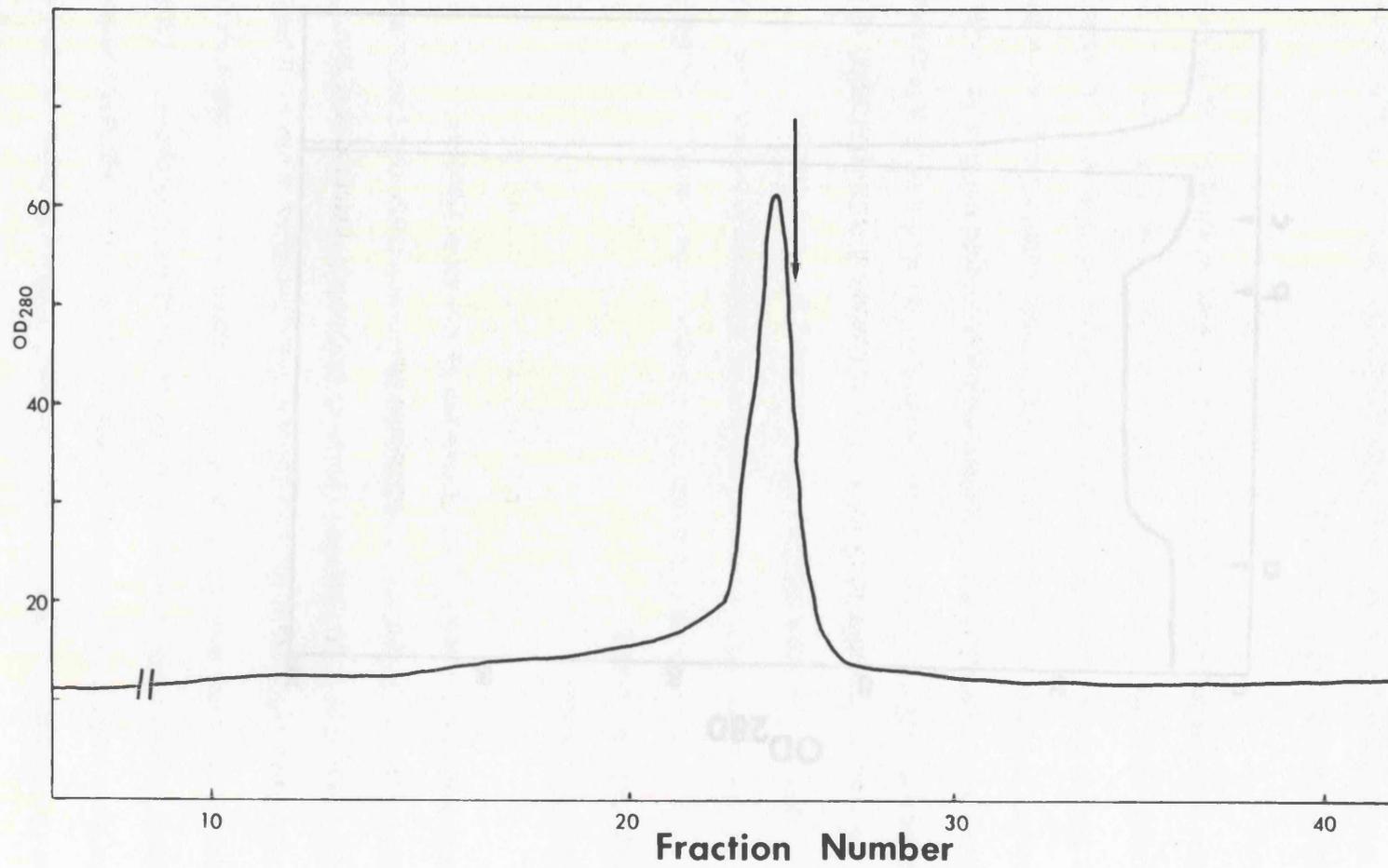


Figure 15 Chromatofocusing column

Trace of optical density at 280 nm, against fraction number of pH 9.4 -7 chromatofocusing column. 50 mls of supernatant following overnight dialysis in 0.5M KCl at pH4.8 and centrifugation at 48,000 g for 2 hours were loaded onto the column and eluted with Polybuffer at pH 7. 3 ml fractions were collected. The trace shows that transgelin is eluted from the column in a single peak, at pH 8 (arrowhead)

Figure 16 Hydroxylapatite Column

Trace of optical density at 280nm against fraction number of hydroxylapatite column. The fraction containing transgelin peak from chromatofocusing column was loaded onto the column, which was washed back to baseline levels, and transgelin was eluted in a single peak with 100mM K phosphate buffer.



13 Actin Preparation

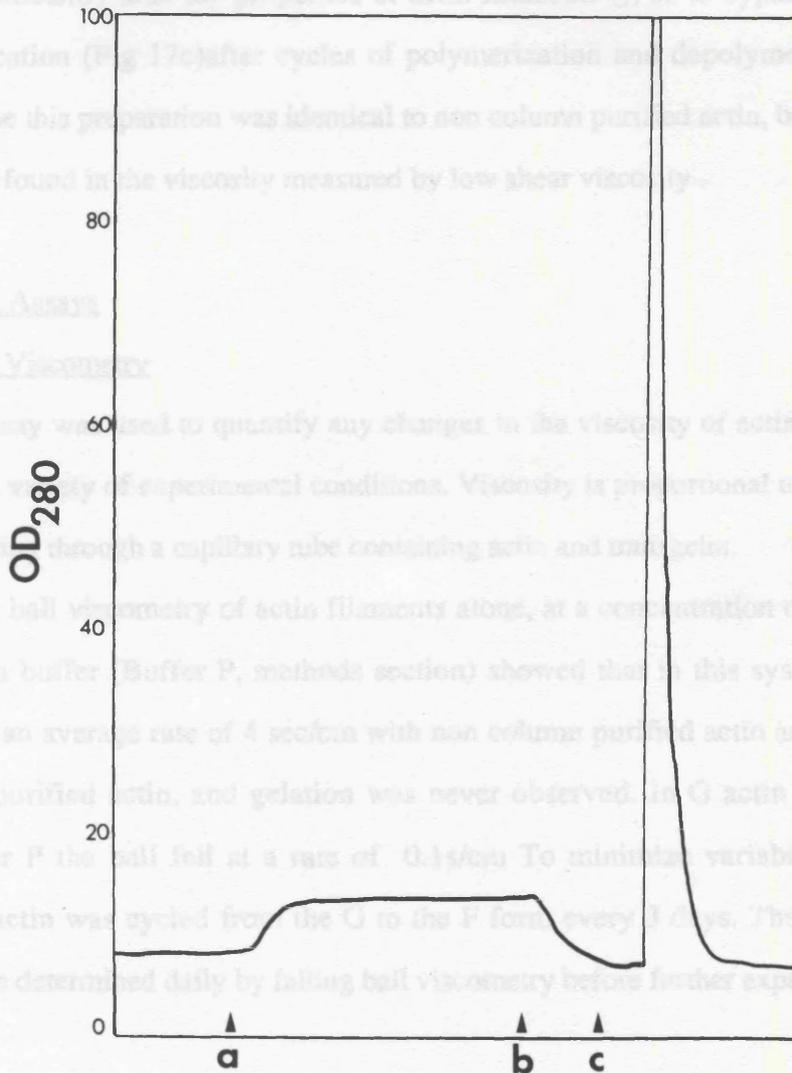
Extraction of actin from an acetone powder preparation of rabbit skeletal muscle yielded 10-30 mg pure actin/g starting material. Two methods of actin preparation were used for these studies. Both utilized the extraction of actin from acetone powder to yield an apparently homogeneous protein preparation as visualized by Coomassie Blue (Fig 17b). However, it has been well described that contaminants at a concentration of 0.15% or lower can significantly alter the properties of actin filaments (3), so to bypass this I used column purification (Fig 17c) after cycles of polymerization and depolymerization. By Coomassie Blue this preparation was identical to non column purified actin, but a dramatic difference was found in the viscosity measured by low shear viscosity.

14 Functional Assays

(a) Falling Ball Viscometry

This assay was used to quantify any changes in the viscosity of actin induced by transgelin, in a variety of experimental conditions. Viscosity is proportional to the velocity of the ball bearing through a capillary tube containing actin and transgelin.

Falling ball viscometry of actin filaments alone, at a concentration of 9.3 μ M, in polymerization buffer (Buffer P, methods section) showed that in this system, the ball bearing fell at an average rate of 4 sec/cm with non column purified actin and 30 sec/cm with column purified actin, and gelation was never observed. In G actin preparations without Buffer P the ball fell at a rate of 0.1 sec/cm. To minimize variability between experiments, actin was cycled from the G to the F form every 1 day. The viscosity of actin alone was determined daily by falling ball viscometry before further experiments were carried out.



(b) Effect of Transgelin Concentration

When varying amounts of purified transgelin, from 0 - 4.2 μ M, were added to 9.3 μ M monomeric actin, under polymerizing conditions, the viscosity of the actin increased, and gelation, that is when the ball bearing remained stationary immediately beneath the meniscus, occurred at a concentration of 2.4 μ M transgelin. Higher transgelin concentrations had no

13 Actin Preparation

Extraction of actin from an acetone powder preparation of rabbit skeletal muscle yielded 10 - 30 mg pure actin / g starting material. Two methods of actin preparation were used for these studies. Both utilized the extraction of actin from acetone powder to yield an apparently homogenous protein preparation as visualised by Coomassie Blue (Fig 17b). However, it has been well described that contaminants at a concentration of 0.15% or lower can significantly alter the properties of actin filaments (), so to bypass this I used column purification (Fig 17c) after cycles of polymerization and depolymerization. By Coomassie Blue this preparation was identical to non column purified actin, but a dramatic difference was found in the viscosity measured by low shear viscosity .

14 Functional Assays

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This assay was used to quantify any changes in the viscosity of actin, induced by transgelin, in a variety of experimental conditions. Viscosity is proportional to the velocity of the ball bearing through a capillary tube containing actin and transgelin.

Falling ball viscometry of actin filaments alone, at a concentration of 9.3 μM , in polymerization buffer (Buffer P, methods section) showed that in this system, the ball bearing fell at an average rate of 4 sec/cm with non column purified actin and 30 sec/cm with column purified actin, and gelation was never observed. In G actin preparations without Buffer P the ball fell at a rate of 0.1s/cm To minimize variability between experiments, actin was cycled from the G to the F form every 3 days. The viscosity of actin alone was determined daily by falling ball viscometry before further experiments were carried out.

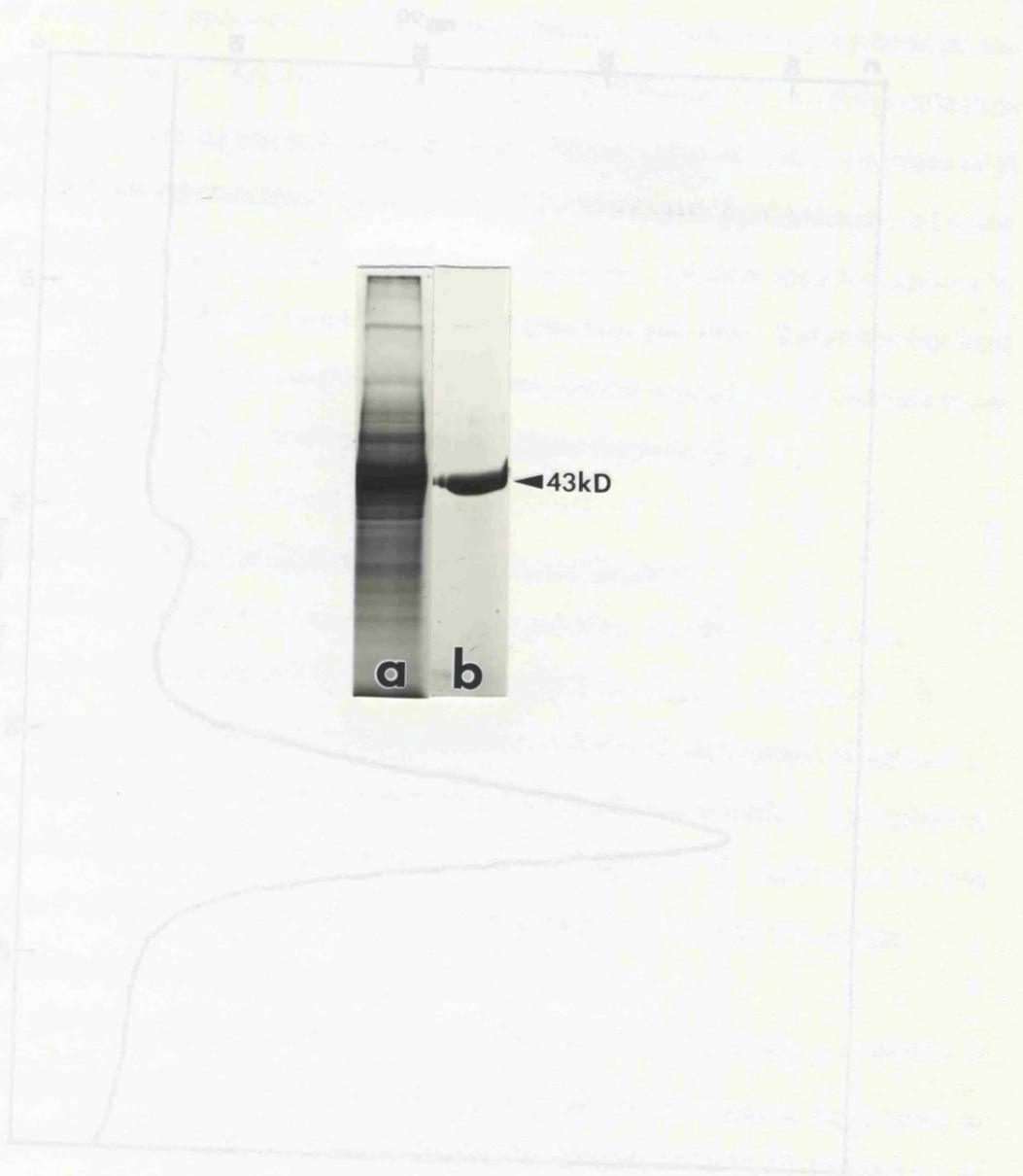
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Figure 17 Purification of actin from rabbit skeletal muscle

Coomassie blue stained tracks of total acetone powder preparation (a) and purified actin (b).

Trace of G actin peak eluted off a Sepharose G150 column with G Buffer (c, overleaf)



effect. The increase from a liquid through which the ball-bearing moved quite rapidly to a gel, where the ball-bearing was completely retarded, was sharp (Fig. 18).¹⁰ However, for the

(b) Pure Culture of Transmembrane Induced Actin Gelation

In a control experiment, purified filaments at $9.3 \mu\text{M}$ were polymerized, and the apparent viscosity measured at intervals after the initiation of the polymerization reaction. The ball-bearing fell at an average rate of 30 μm after an initial lag phase of at least 1 minute. This lag remained constant even after 30 minutes, and gelation was never observed (Fig. 19).¹⁰ However, the addition of transgelin at a concentration known to induce actin gelation (2.4 μM) caused the actin filaments to gel within 3 minutes, but here there was an initial lag phase of at least 1 minute, during which there was increase in the apparent viscosity of the α - β -transgelin was essentially the same (Fig. 19).¹⁰

(c) Effect of Transmembrane on Transmembrane Induced Actin Gelation

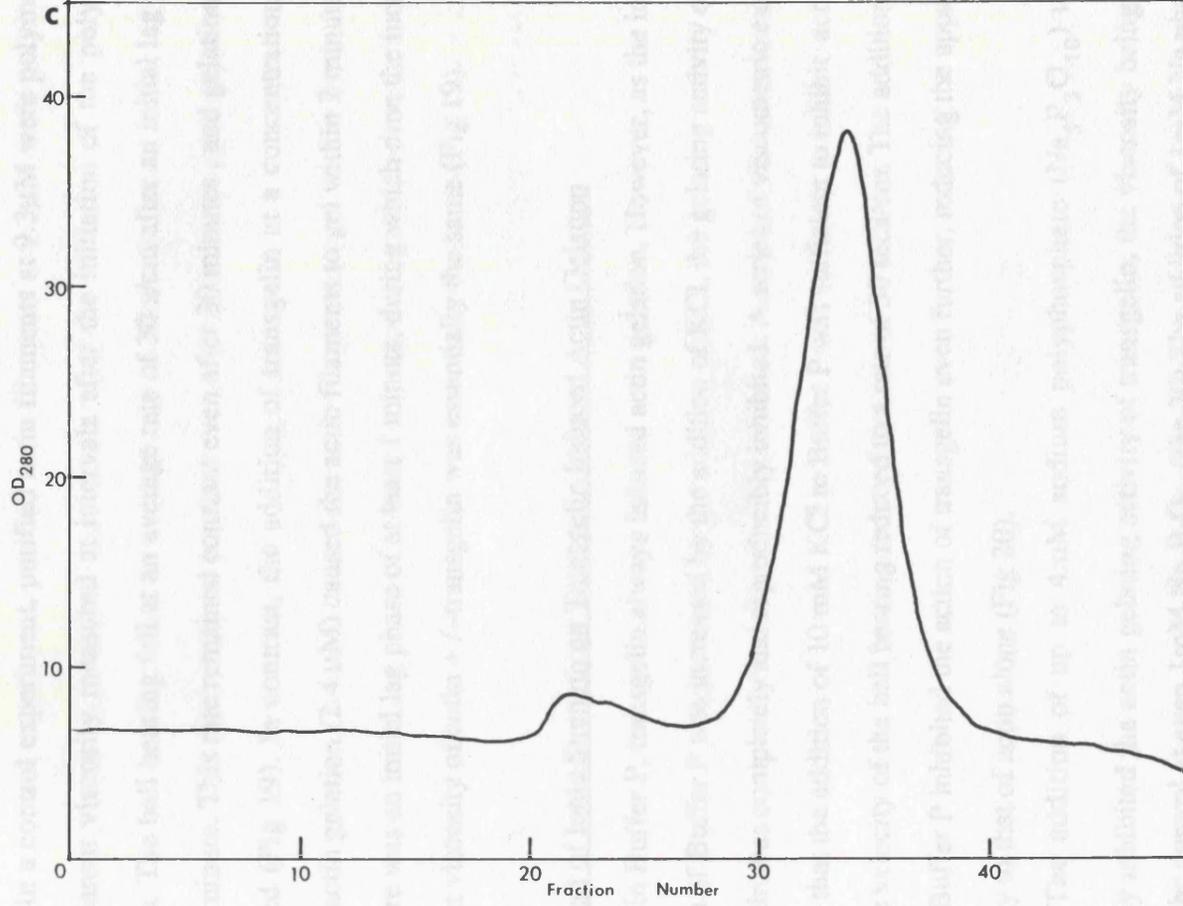
Buffer P, transgelin always induced actin gelation. Moreover, as the ionic strength of Buffer P was increased by the addition of KCl, the gelating activity of transgelin was completely inhibited. A series of viscosimetric experiments showed that the addition of 10 mM KCl to Buffer P completely inhibited actin gelation, with the viscosity of the ball-bearing reduced to that of a liquid. The addition of 30 mM KCl to Buffer P inhibited the action of transgelin even further, reducing the apparent viscosity of actin alone (Fig. 20).¹⁰

The addition of up to 4.0M sodium polyphosphate ($\text{Na}_2\text{P}_2\text{O}_7$) to Buffer P invariably inhibited the action of transgelin, the viscosity being reduced to that of the

no effect on transgelin induced actin gelation.

(d) Effect of Pronounced G-actin with Transmembrane on Transmembrane Induced Gelation

G-actin, in the absence of Buffer P, no induced polymerization, did not retard the ball-bearing at all (less than 1 sec/cm). This was also found with G-actin and transmembrane in the absence of Buffer P were used in the assay system.



effect. The transition from a liquid, through which the ball bearing moved quite rapidly to a gel, where the ball bearing was completely retarded, was sharp (Fig 18).

(b) Time Course of Transgelin Induced Actin Gelation

In a control experiment, purified actin filaments at $9.3\mu\text{M}$ were polymerised, and the apparent viscosity measured at intervals after the initiation of the polymerization reaction. The ball bearing fell at an average rate of 30 s/cm after an initial lag phase of at least 1 minute. This rate remained constant even after 30 minutes, and gelation was never observed (Fig 19). In contrast, the addition of transgelin at a concentration known to induce actin gelation ($2.4\mu\text{M}$) caused the actin filaments to gel within 2 minutes, but here too, there was an initial lag phase of at least 1 minute, during which time the increase in the apparent viscosity of actin + / -transgelin was essentially the same (Fig 19).

(c) Effect of Ionic Strength on Transgelin Induced Actin Gelation

In Buffer P, transgelin always induced actin gelation. However, as the ionic strength of Buffer P was increased by the addition of KCl, the gelating activity of transgelin was completely and reproducibly inhibited. A series of viscometric experiments showed that the addition of 10 mM KCl to Buffer P was sufficient to inhibit actin gelation, with the velocity of the ball bearing reduced to a rate of 50 secs/cm. The addition of 30 mM KCl to Buffer P inhibited the action of transgelin even further, reducing the apparent viscosity to that of actin alone (Fig 20).

The addition of up to 4mM sodium polyphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$) to Buffer P similarly inhibited the actin gelating activity of transgelin, the viscosity being reduced to that of the control at even 1mM $\text{Na}_5\text{P}_3\text{O}_{10}$ (Fig 20). The addition of 1mM Na phosphate had no effect on transgelin induced actin gelation.

(d) Effect of Preincubating G actin with Transgelin on Transgelin Induced Gelation

G actin, in the absence of Buffer P to induce polymerization, did not retard the ball bearing at all (less than 1sec/5cm) This was also found when G actin and transgelin in the absence of Buffer P were used in the assay system.

Pre-incubation of transgelin with G actin monomers before the initiation of polymerization did not inhibit the ability of transgelin to induce actin gelation. In the presence of transgelin, gelation had occurred within 5 minutes after the initiation of polymerization, while actin alone did not gel.

(e) Effect of Transgelin on Actin Filament Annealing

G actin was induced to form filaments by the addition of Buffer P. After a 30 minute incubation period these polymers were fragmented, and apparent viscosity measured at intervals after the addition of transgelin.

The transgelin induced gelation of actin fragments was slower than that seen with actin monomers, gelation was not seen within 5 minutes but had occurred by 30 minutes. However, after 5 minutes, the viscosity of actin + transgelin was almost 3 times that of actin alone. Actin fragments annealing without transgelin did show an increase in viscosity over the 30 minute period, but did not form a gel.

(f) Effect of pH on Transgelin Induced Actin Gelation

Variations in pH from 5 - 10 had no effect on transgelin induced actin gelation, which always occurred at the same transgelin concentration. Actin gelation was always seen in the presence of transgelin, never in its absence.

(g) Effect of Calcium on Transgelin Induced Actin Gelation

A range of calcium concentrations, from 0.05 to 1.5mM had no effect on transgelins ability to induce actin gelation. The removal of Ca^{2+} , by the addition of up to 2mM EGTA similarly had no effect on transgelin induced actin gelation. Actin, in the presence of 9.3 μM transgelin, forms a gel in the absence or presence of Ca^{2+} or EGTA. Actin alone, at a concentration of 9.3 μM , in the presence or absence of Ca^{2+} or EGTA never formed a gel and has an average viscosity of 30 sec/cm.

Figure 18 Effect of transgelin on actin viscosity

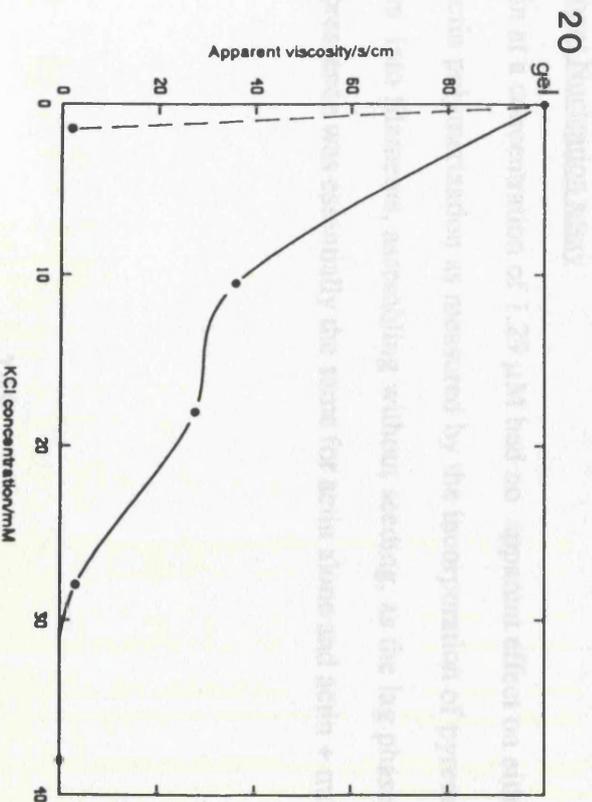
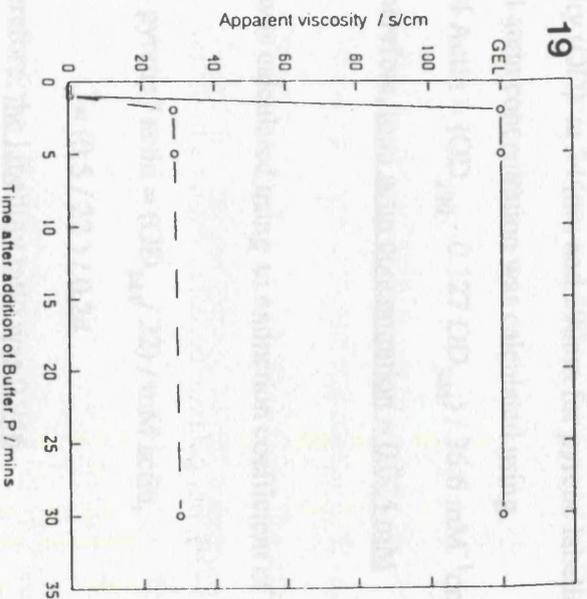
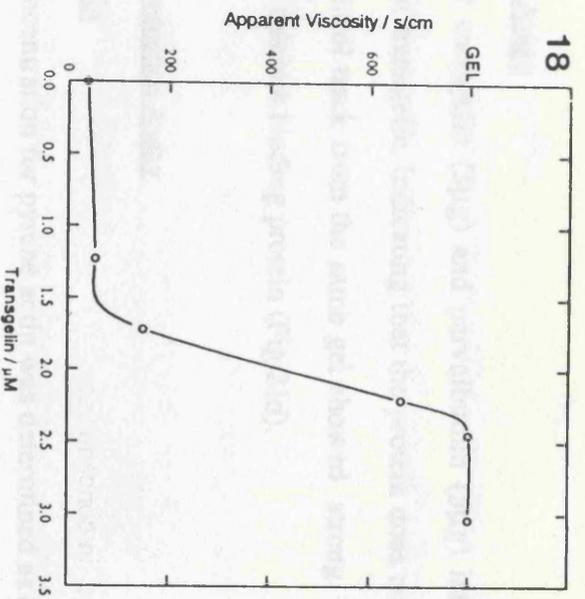
G actin ($9.3 \mu\text{M}$) was incubated with varying concentrations of transgelin in Buffer P in a capillary tube. Apparent viscosity (which is proportional to the velocity of the ball bearing through a capillary tube containing the solution of actin and transgelin) was measured after a 30 minute incubation period. Actin gelation (defined here as viscosity at which the ball bearing remaining stationary immediately beneath the meniscus) was induced at a concentration of $2.4 \mu\text{M}$ transgelin. A sharp transition from viscous liquid to gel occurs as a function of transgelin concentration.

Figure 19 Time course of actin gelation +/- transgelin

Apparent viscosity was measured at various times after the addition of a concentration of transgelin known to induce actin gelation ($2.4 \mu\text{M}$) to G actin ($9.3 \mu\text{M}$) in Buffer P. After an initial lag period of 1 minute when there was no detectable increase in apparent viscosity, the viscosity of actin alone (-----) increased to 30 s/cm by 2 minutes, and did not change after 30 minutes. A similar lag period of 1 minute was seen in the presence of transgelin (————), but gelation occurred within 2 minutes.

Figure 20 Ionic strength control of transgelin induced actin gelation

G actin ($9.3 \mu\text{M}$) was incubated with transgelin ($2.4 \mu\text{M}$) in Buffer P + various concentrations of KCl (————) up to 40mM. The addition of 10 mM KCl was sufficient to inhibit transgelin induced actin gelation, reducing the apparent viscosity to 50 s/cm. The addition 30 mM KCl reduced the apparent viscosity of actin to that of actin alone. The addition of 1mM $\text{Na}_5\text{PO}_3\text{O}_{10}$ (-----) to Buffer P inhibited transgelin induced actin gelation. These values are normalized with respect to actin controls. 1mM $\text{Na}_5\text{P}_3\text{O}_{10}$.



ii ⁴⁵Calcium Binding Assay

Immunoblots of transgelin (5 μ g) and parvalbumin (5 μ g) labelled with ⁴⁵Ca²⁺ showed no labelling of transgelin, indicating that the protein does not bind ⁴⁵Ca²⁺ (Fig 21c) In contrast, a control track from the same gel showed strong ⁴⁵Ca²⁺ labelling of parvalbumin, a known calcium binding protein (Fig 21d).

iii Pyrene Actin Fluorescence Assay

(a) Pyrene labelled Actin

The protein concentration for pyrene actin was determined as outlined (Bryan and Coluccio).

The optical density (OD) at 344nm and 290nm for pyrene labelled actin was 0.5 and 0.72 respectively. Total actin concentration was calculated using

$$\text{mM Actin} = (\text{OD}_{290} - 0.127 \text{OD}_{344}) / 26.6 \text{ mM}^{-1}\text{cm}^{-1}$$

Therefore, total actin concentration = 0.024 mM

The pyrene:actin ratio was calculated using an extinction coefficient of 22,000 M⁻¹cm⁻¹ for pyrene with

$$\begin{aligned} \text{pyrene / actin} &= (\text{OD}_{344} / 22) / \text{mM actin}, \\ &= (0.5 / 22) / 0.24 \end{aligned}$$

Therefore, the labelling ratio was 0.946

(b) Actin Filament Nucleation assay

Transgelin at a concentration of 1.29 μ M had no apparent effect on either the onset or the rate of actin polymerization as measured by the incorporation of pyrene labelled G actin monomers into filaments, assembling without seeding, as the lag phase and rate of increase of fluorescence was essentially the same for actin alone and actin + transgelin (Fig 22).

(c) Actin Filament Elongation Assay

The rate at which G actin monomers were incorporated onto preformed actin filaments was unaltered by the presence or absence of transgelin (Fig 23).

iv 90° Light Scattering

When 2.4 μM transgelin was added to 9.3 μM actin in polymerising conditions, a rapid increase in 90° light scattering was seen, which was 60 fold greater than control levels (actin alone) within 3 - 4 minutes.

This effect was totally abrogated when the reaction was carried out in the presence of 100mM KCl, with the 90° light scattering of actin + transgelin reduced to the level of controls (Fig 24).

v Electron Microscopy

Actin filaments, formed by the polymerisation of G actin for 10 minutes in Buffer P and then examined by negative stain electron microscopy, formed a random loose meshwork over the entire grid, with no clear area visible (Fig 25a). In contrast, actin polymerised for only 1 minute but in the presence of 2.4 μM transgelin was aggregated into large crosslinked tangles between which clear areas of grid, devoid of actin filaments, were visible (Fig 25b).

When actin and transgelin were incubated together for 10 minutes, in polymerising conditions, the tangles seen in Fig 25b were tightly aggregated and crosslinked into smaller, discrete foci (Fig 25c), in which individual actin filaments could be discerned only at the edges (Fig 25d).

Fig 21 $^{45}\text{Ca}^{2+}$ Binding

Coomassie blue stained tracks of purified transgelin (a) (asterisk) and parvalbumin (b) (arrowhead). Immunoblot of tracks (a) and (b) incubated with $^{45}\text{Ca}^{2+}$ show the absence of $^{45}\text{Ca}^{2+}$ binding to transgelin (c) while parvalbumin is strongly labelled (d).

21

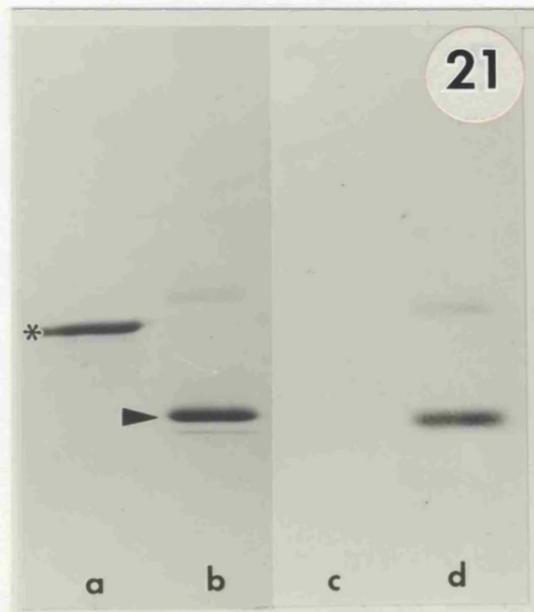
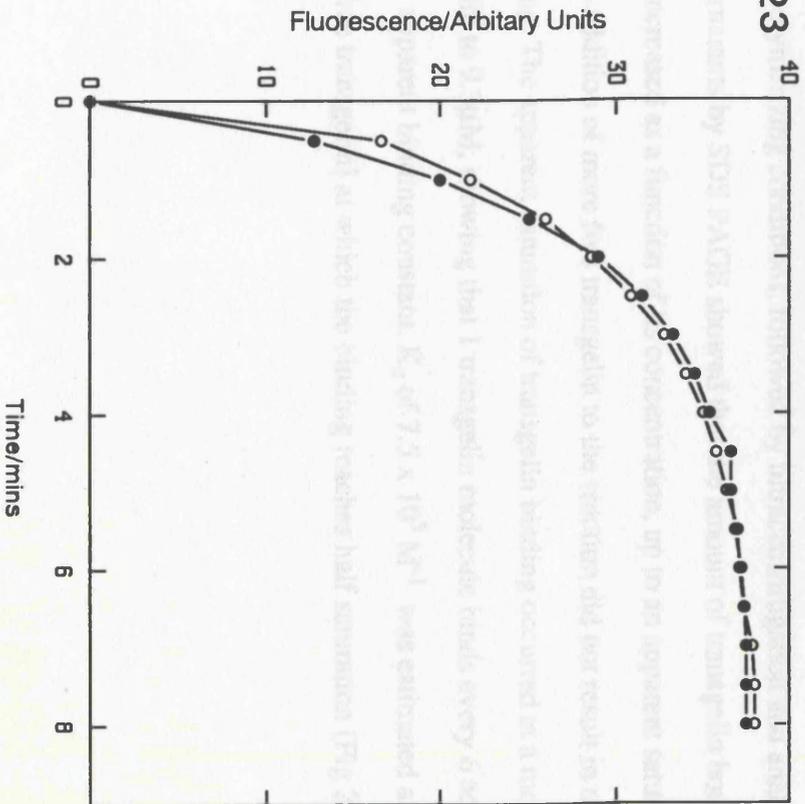
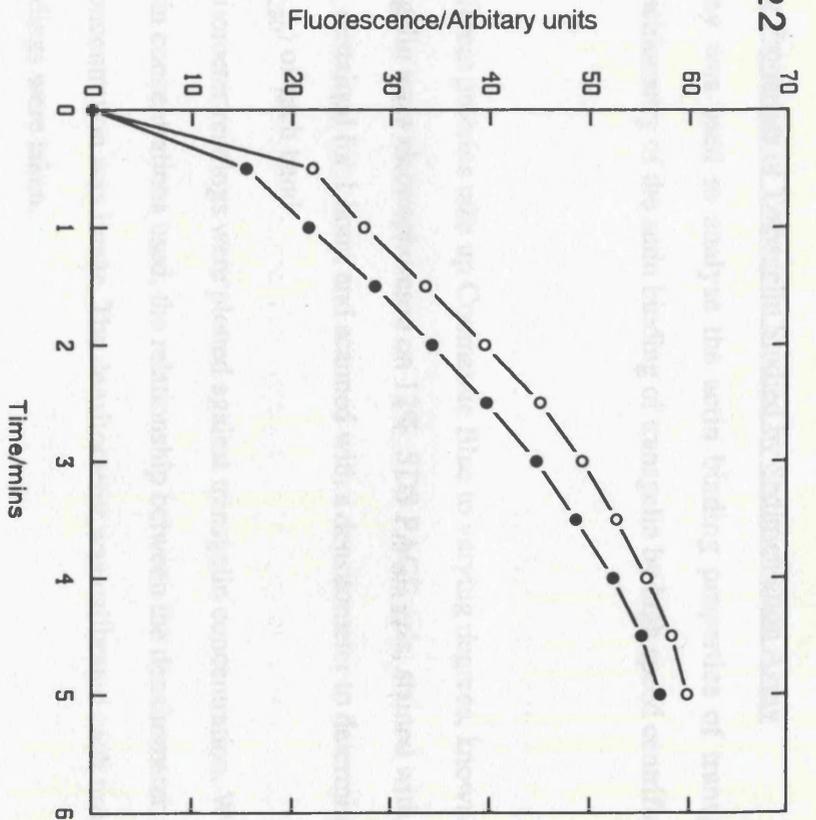


Figure 22 Effect of transgelin on actin nucleation

Actin ($4\mu\text{M}$) + $1\mu\text{M}$ pyrene labelled actin was induced to polymerise by incubation in Buffer P, + (—○—) or - (—●—) $1.29\mu\text{M}$ transgelin. Transgelin had no apparent effect on either the onset, or the rate of filament formation.

Figure 23 Effect of transgelin on actin filament elongation

Actin filaments were preformed by incubating $5\mu\text{M}$ actin in Buffer P for 30 minutes at 25°C in the presence (—○—) or absence (—●—) of $1.29\mu\text{M}$ transgelin. The effect of transgelin on filament elongation was determined by measuring the change in fluorescence that occurred during the subsequent incorporation of G actin monomers ($4\mu\text{M}$ + $1\mu\text{M}$ pyrene labelled actin). Transgelin has no effect on the elongation rate of actin filaments.



15 Actin Binding Properties of Transgelin Studied by Sedimentation Assay

This assay was used to analyse the actin binding properties of transgelin. It measured the stoichiometry of the actin binding of transgelin by high speed centrifugation.

i Calibration

Since different proteins take up Coomassie Blue to varying degrees, known amounts of transgelin were electrophoresed on 12% SDS PAGE gels, stained with Coomassie Blue, destained for 1 hour, and scanned with a densitometer to determine the absorbance (OD_{280}) of each band .

The densitometer readings were plotted against transgelin concentration. Within the range of transgelin concentrations used, the relationship between the densitometer readings and transgelin concentration was linear. The densitometer was calibrated each time before experimental readings were taken.

ii Determination of Binding Constant

Incubation of purified transgelin at concentrations between 0.6 and 12 μ M with 9.3 μ M actin in polymerizing conditions, followed by ultracentrifugation and analysis of pellets and supernatants by SDS PAGE showed that the amount of transgelin bound to F actin filaments increased as a function of its concentration, up to an apparent saturation point, where the addition of more free transgelin to the reaction did not result in more transgelin binding. The apparent saturation of transgelin binding occurred at a molar ratio of 1 μ M transgelin to 9.3 μ M, showing that 1 transgelin molecule binds every 6 actin monomers. The apparent binding constant, K_a of $7.5 \times 10^5 \text{ M}^{-1}$ was estimated as the inverse of the [free transgelin] at which the binding reaches half saturation (Fig 26h).

Figure 24 Actin filament crosslinking by transgelin

The increase in light scattering by actin filaments when $9.3\mu\text{M}$ actin was induced to polymerize by addition of Buffer P + (\circ) or - (∇) 100mM KCl in the absence of transgelin was measured and compared with actin ($9.3\mu\text{M}$) induced to polymerise in Buffer P + (\blacktriangledown) or - (\bullet) 100mM KCl in the presence of $2.4\mu\text{M}$ transgelin. The rapid increase in light scattering when actin polymerised in Buffer P + transgelin (\bullet) was not seen in Buffer P + 100mM KCl.

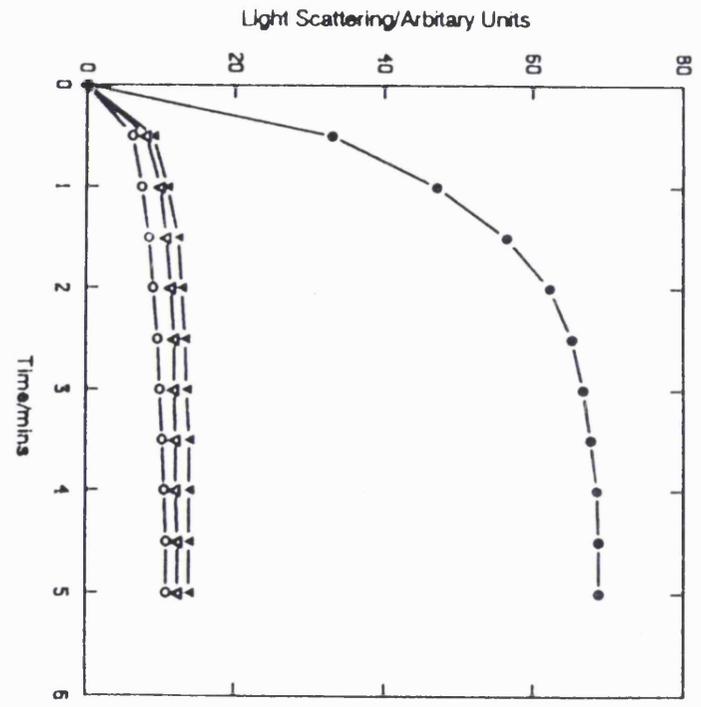


Figure 25 Negative stain electron microscopy of actin filaments +/- transgelin

(a) Actin ($0.5\mu\text{M}$) polymerized for 10 minutes in Buffer P shows an essentially random, loose network of filaments which covers the grid.

(b) Actin ($0.5\mu\text{M}$) was polymerised for 1 minute in Buffer P + $2.4\mu\text{M}$ transgelin. Actin filaments are tangled and form loose aggregates. Large areas of the grid are devoid of actin filaments.

(c) Actin ($0.5\mu\text{M}$) was polymerised for 10 minutes in Buffer P + $2.4\mu\text{M}$ transgelin. The actin tangles have become tightly aggregated into small dense foci, in which individual actin filaments are visible at the edges.

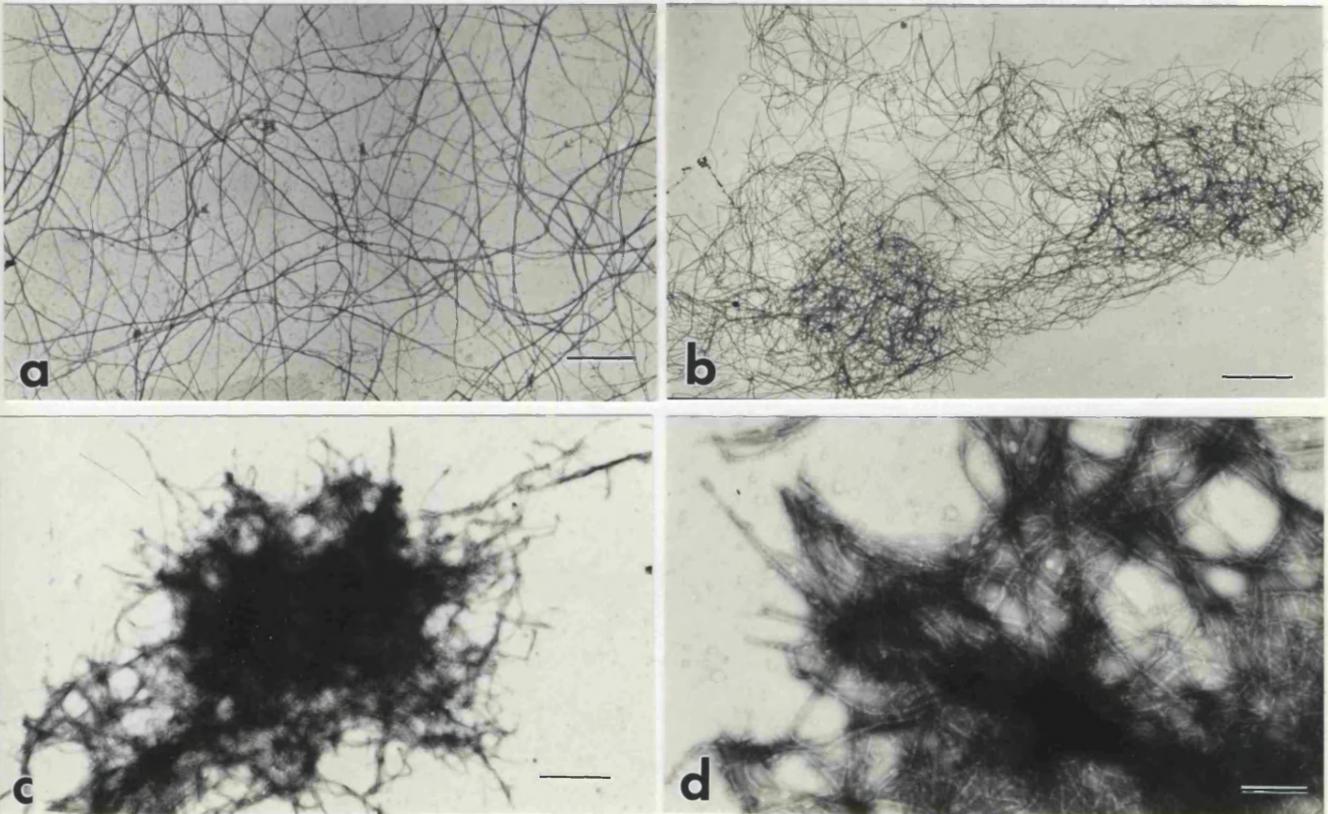
(d) High power image of (c) shows the dense meshwork of individual actin filaments that form the foci.

Bars (a and d) $25\mu\text{M}$ (b) $2.8\mu\text{M}$ and (c) $0.8\mu\text{M}$

Controls indicate that the amount of actin pelleted was the same under all conditions and that transgelin was not pelleted without actin present. To minimize variation in staining between gels, both supernatants and pellets in one series of experiments were run on the same SDS PAGE gel (Fig. 26 a-h).

16. Liver Actin and Transgelin Gradients

Liver actin gradients showed that transgelin remained at a constant banding at



Controls indicate that the amount of actin pelleted was the same under all conditions and that transgelin was not pelleted without actin present. To minimize variation in staining between gels, both supernatants and pellets in one series of experiments were run on the same SDS PAGE gel (Fig 26 a-h).

16 Linear Sucrose Density Gradients

Linear sucrose gradients showed that transgelin remained as a monomer, banding at an identical position to SBTI, a monomeric protein of 21kDa in ionic conditions which are known to either promote (Buffer P) or prevent (Buffer P +100mM KCl) the actin binding/gelating activity of the molecule. In further control gradients, run in parallel, ovalbumin, bovine serum albumin and transferrin with well characterised molecular masses of 43, 68 and 80 kD respectively were loaded. These distinctly and reproducibly banded at lower points (that is higher sucrose densities) in both high and low salt gradients (Fig 27).

Figure 26 Binding constant of transgelin to actin

G actin (9.3 μ M) was incubated for 30 minutes at 25 $^{\circ}$ C in Buffer P with increasing concentrations of transgelin. After centrifugation at 148,000 g for 1 hr in a Beckman Airfuge, supernatants and pellets were resuspended in equal volumes of SDS sample buffer and analysed on 12% SDS PAGE gels (tracks a - f), prestained molecular weight markers (g) Protein concentrations were determined by densitometry. The apparent binding constant of transgelin to actin, K_a , was estimated to be $7.5 \times 10^5 \text{ M}^{-1}$. Saturation of binding was reached at a molar ratio of 1 transgelin molecule: 6 actin monomers (h).

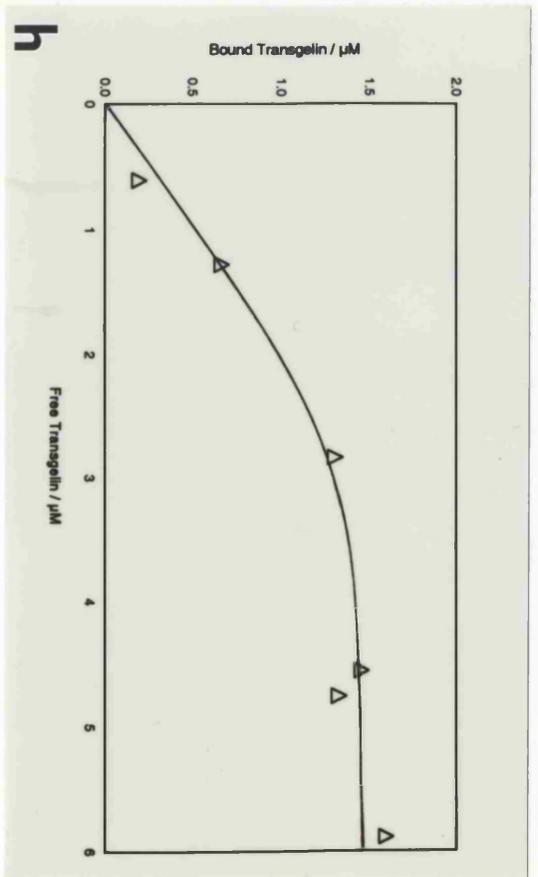


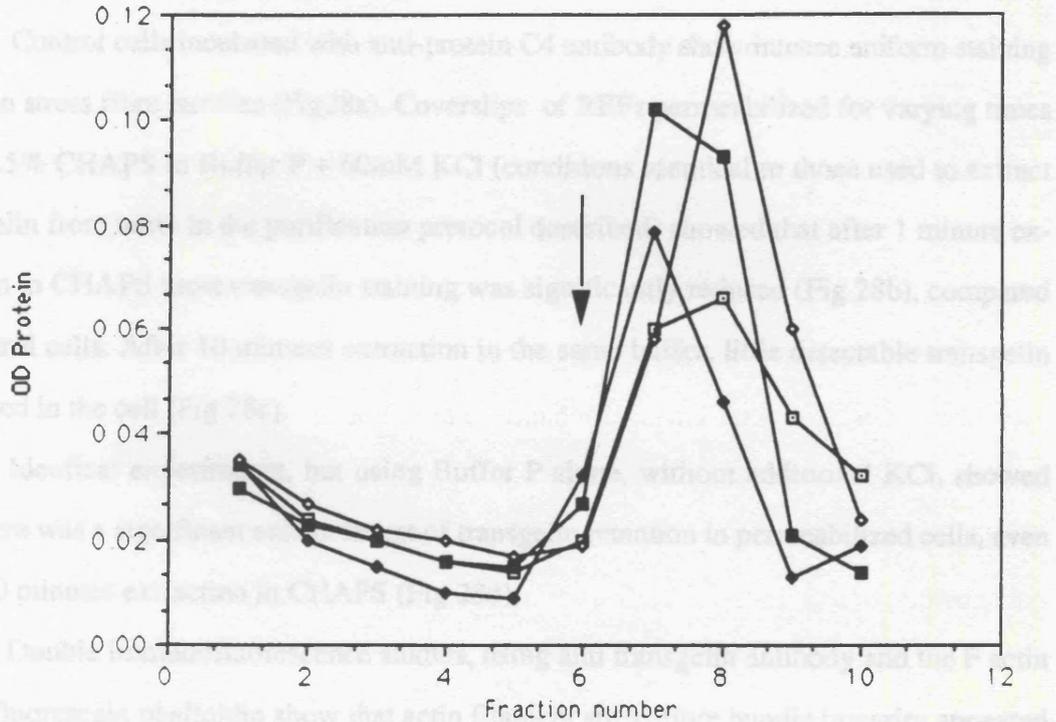
Figure 27 Linear Sucrose density gradient centrifugation of transgelin

Purified transgelin in 12mM Buffer P —■— was layered on to a 5-20% linear sucrose gradient in the same buffer and centrifuged at 150,000 g for 43 hours at 4°C. Soy bean trypsin inhibitor in Buffer P —◆—, transgelin in Buffer P + 100mM KCl —◇—, and soy bean trypsin inhibitor in Buffer P +100mM KCl —□— were run in parallel gradients. Transgelin bands at the same level as soy bean trypsin inhibitor, which has an equivalent Mr of 21 kD, in both Buffer P and Buffer P + 100 mM KCl, indicating that transgelin remains as a monomer in these ionic conditions. Arrow marks the position of ovalbumin (43 kDa).

17 Immobilized Cell Assays

This assay was developed to further investigate the characteristics of purified transgelin and to obtain positional information about its rebinding *in vivo*.

i. Detergent Removal of Transgelin



to be unaffected (Fig 28g overall).

ii. Transgelin Binding in Extruded Cells

When purified transgelin, at a concentration of $1 \mu\text{M}$ in Buffer P + $50 \mu\text{M}$ KCl was added to cells prepared in the same buffer for 15 minutes, so that all detectable endogenous transgelin had been removed, very little transgelin rebinding was ever seen compared with control cells (Fig 28e).

However, when purified transgelin at a concentration of $2 \mu\text{M}$ in Buffer P alone was added to similarly extruded RBFs, an increase in transgelin rebinding to levels indistinguishable from control

cells was found. In these cells, rebound transgelin was found only on actin stress fibre bundles, where it was seen along the entire length of the stress fibres (Fig 28f).

17 Permeabilized Cell System

This assay was developed to further investigate the characteristics of purified transgelin and to obtain positional information about its rebinding *in vivo*.

i Detergent Removal of Transgelin

Control cells incubated with anti-protein C4 antibody show intense uniform staining on actin stress fibre bundles (Fig 28a). Coverslips of REFs permeabilized for varying times with 0.5% CHAPS in Buffer P + 60mM KCl (conditions identical to those used to extract transgelin from aorta in the purification protocol described) showed that after 1 minute extraction in CHAPS most transgelin staining was significantly reduced (Fig 28b), compared to control cells. After 10 minutes extraction in the same buffer, little detectable transgelin remained in the cell (Fig 28c).

Identical experiments, but using Buffer P alone, without additional KCl, showed that there was a significant enhancement of transgelin retention in permeabilized cells, even after 10 minutes extraction in CHAPS (Fig 28d).

Double immunofluorescence studies, using anti transgelin antibody and the F actin probe fluorescein phalloidin show that actin filament stress fibre bundle integrity appeared to be unaffected (Fig 28 g overleaf).

ii Transgelin Rebinding to Permeabilized Cells

When purified transgelin, at a concentration of 3 μ M in Buffer P + 60mM KCl was added to cells permeabilized in the same buffer for 15 minutes, so that all detectable endogenous transgelin had been removed, very little transgelin rebinding was ever seen compared with control cells (Fig 28e).

However, when purified transgelin at a concentration of 3 μ M in Buffer P alone was added to similarly extracted REFs, an increase in transgelin rebinding to levels indistinguishable from control cells was found. In these cells, rebound transgelin was found only on actin stress fibre bundles, where it was seen along the entire length of the stress fibres (Fig 28f).

Figure 28 Ionic dependence of transgelin removal and rebinding in detergent permeabilised REFs.

(a) Controls of methanol permeabilised REFs labelled with anti protein C4 and anti mouse IgG/Rhodamine show that transgelin is distribution on actin stress fibre bundles. All other micrographs were photographed using the same exposure as for this cells.

(b) REFs permeabilised for 1 min in 0.5% CHAPS in Buffer P + 60mM KCl and labelled as in (a) show that most protein C4 has been removed.

(c) REFs permeabilised as above but for 10 minutes show little detectable protein C4 labelling.

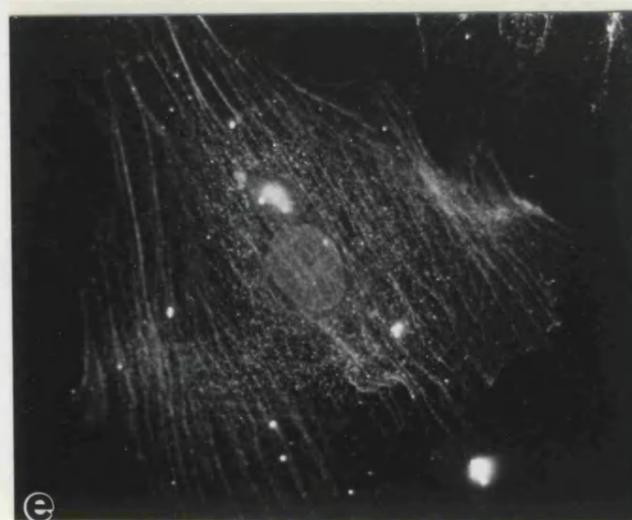
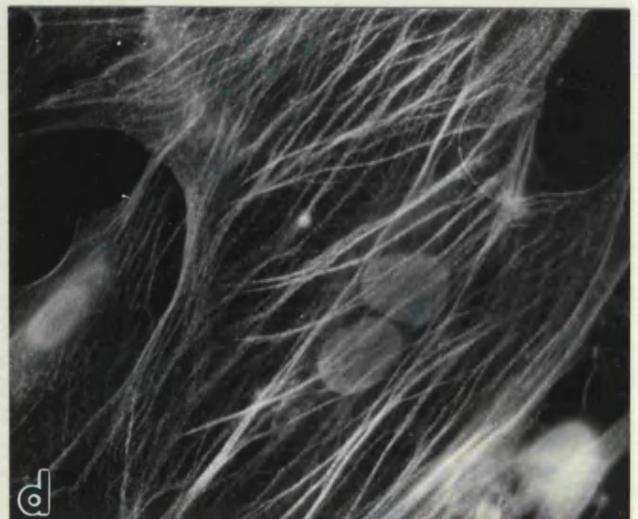
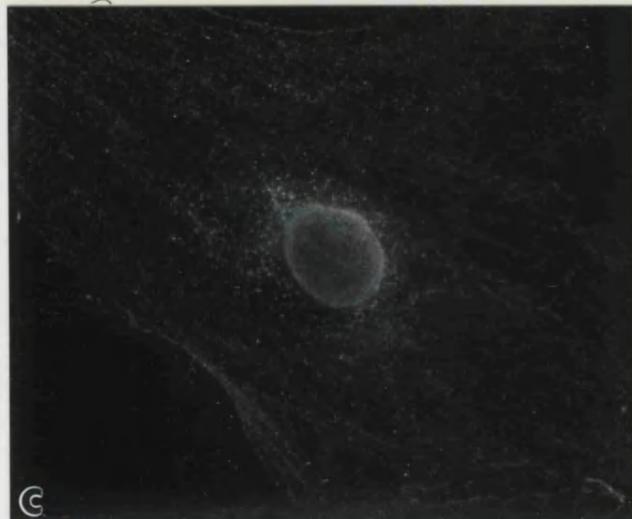
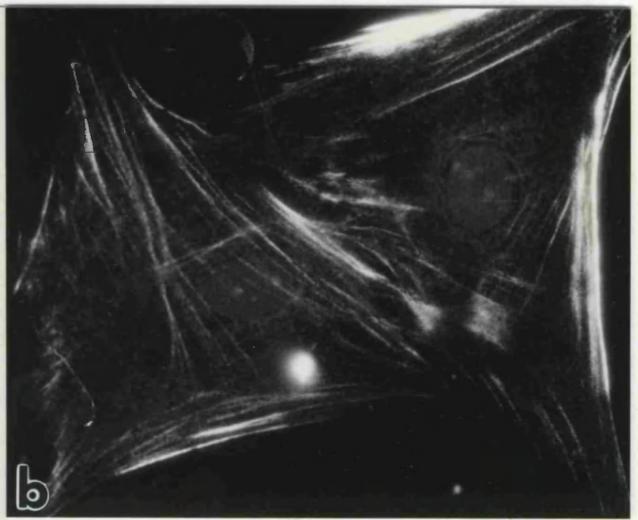
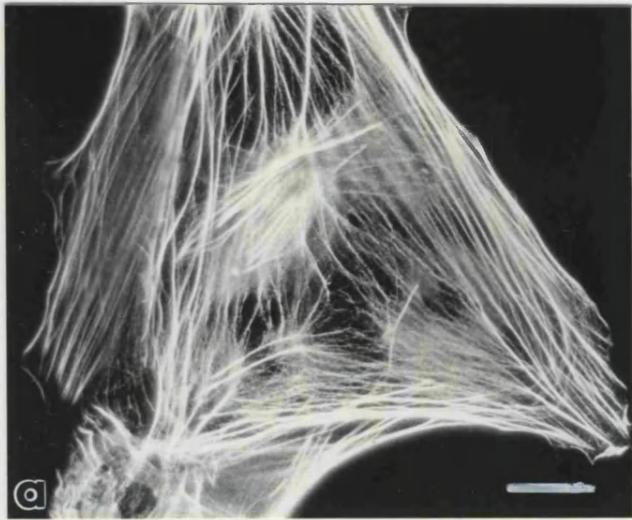
(d) REFs permeabilised for 10 minutes in 0.5% CHAPS in Buffer P without KCl show that a significant amount of protein C4 is retained in these cells.

(e) REF detergent extracted as in (c) to remove detectable protein C4, and then incubated with 3 μ M transgelin in Buffer P + 60mM KCl show that very little transgelin rebinds in these cells.

(f) REFs permeabilised as in (c) and then incubated with 3 μ M transgelin in Buffer P alone shows that transgelin rebinds to actin stress fibre bundles in these cells. The amount of transgelin rebinding is indistinguishable from the control cell in (a).

(g) Phalloidin labelled REFs after extraction as in (c) show that actin stress fibre bundles were unaffected.

Bar equals 20 μ m.

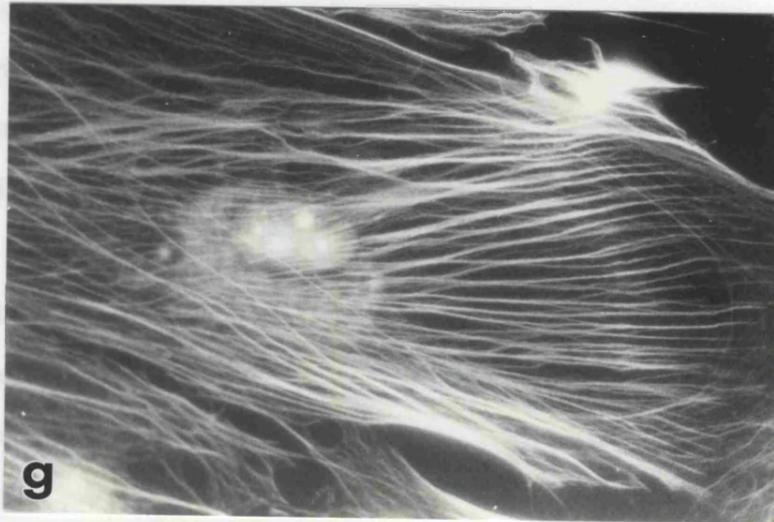


DISCUSSION

I have adopted the same nomenclature for protein C4 as in the results section, namely that the purified higher molecular weight isoform is designated transgelin, the lower molecular weight isoform in all instances is called C4', while protein C4 is used to refer to both isoforms expressed together. Both the monoclonal and the polyclonal antibodies recognize both isoforms and are defined as anti-protein C4 antibody.

1. Immunofluorescence

Immunofluorescence experiments in two-dimensional agarose gels and in only structural techniques is a lack of resolution



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2. Protein C4 Distribution in Normal Tissue Culture Cells

At the resolution of the light microscope protein C4 was found to have a continuous, uniform distribution along all major axes of these bundles spanning the cell. To determine whether the intracellular sites recognized by anti-C4 antibody were F-actin stress fibers, the filamentous actin specific probe, phalloidin, was used in double immunofluorescence experiments with anti-protein C4 antibody. Phalloidin is a fungal toxin isolated from *Amanita phalloides* which binds exclusively to F-actin structures with high affinity (46). Phalloidin is a fluorochrome and used in immunofluorescence experiments is found to the same cytoskeletal network as an affinity

DISCUSSION

I have adopted the same nomenclature for protein C4 as in the results section, namely that the purified higher molecular weight isoform is designated transgelin, the lower molecular weight isoform in all instances is called C4^l, while protein C4 is used to refer to both isoforms expressed together. Both the monoclonal and the polyclonal antibodies recognize both isoforms and are defined as anti protein C4 antibody .

1 Immunofluorescence

Immunofluorescence techniques have been widely used in studies of the cytoskeleton. The advantages of this method are the speed and simplicity of the experimental procedures, and the positional information that can be derived from the three dimensional images of the cytoskeletal networks which are produced (249,334,248). A major limitation is that the resolution of the light microscope is 400-700 nm (6), enabling only structures larger this minimum size to be resolved. Another problem inherent with this technique is image superposition, due to light emission from the fluorochrome, leading to a lack of resolution and distortion of the image. Pre crisis REFs were used in all these studies with cultures being discarded after 10 passages to avoid the establishment of cell lines, which become adapted for growth in culture by several phenotypic changes, including alterations to the cytoskeleton.

2. Protein C4 Distribution in Normal Mesenchymal Cells

At the resolution of the light microscope, protein C4 was found to have a continuous, uniform distribution along the entire length of linear bundles spanning the cell. To determine whether the intracellular structures labelled by anti-C4 antibody were F actin stress fibres, the filamentous actin specific probe, fluorescein phalloidin was used in double immunofluorescence experiments with anti-protein C4 antibody. Phalloidin is a fungal toxin isolated from *Amanites phalloides* which binds exclusively to F actin structures with high affinity (461,478). When conjugated to a fluorochrome and used in immunofluorescence experiments it binds to the same cytoskeletal network as an affinity

purified anti-actin antibody (478), and this specificity for F actin has been confirmed by microinjection and *in vivo* (56). The advantages of using fluorescein phalloidin over an anti-actin antibody are (a) increased labelling, since it is a small molecule (Mr 1250) (461,478), and therefore less likely to be excluded from structures that have been cross linked by fixation, (b) lack of diffuse cytoplasmic staining seen with anti-actin antibodies which will also label G actin (461) and (c) loss of non-specific staining which can arise with second antibodies. This could be avoided by use of either an affinity purified polyclonal anti-actin antibody or a monoclonal antibody conjugated directly to a fluorochrome, but anti-actin antibodies are very difficult to raise, due to the need to break immune tolerance, since actin is an abundant protein in all tissues (476,388). In no instance was any area of stress fibre bundles seen that was labelled with phalloidin but not with anti-protein C4 antibody.

In contrast to the uniform distribution of protein C4, described above, many microfilament associated proteins show marked periodicities. For example, myosin is often distributed as nonaligned patches of variable length and spacing or aggregated into larger periodically spaced patches (247) while α actinin and tropomyosin are localized in a periodic alternate fashion along actin filament bundles in cultured cells (249). Caldesmon and tropomyosin show coincident periodic distribution, which may reflect their close functional association in regulating actomyosin interactions (492). It has been suggested that this periodicity may be due to the sarcomere like organization of microfilaments in stress fibres, in which they mimic skeletal muscle myofibrils (56,53). Occasionally this periodicity is poorly resolved in thicker bundles, which may reflect the dynamic process of addition and deletion of actin associated proteins from stress fibres as they form and disappear in cultured cells (56). It also suggests that the length of the striations is variable and that the individual fibres within an actin filament show lateral freedom of movement and can slide relative to each other (56). This is unlikely to be the cause of protein C4 non-periodic distribution, since in all instances, a uniform distribution of protein C4 in stress fibre bundles is seen.

The intensity of anti-protein C4 staining is constant along the length of a stress fibre, the apparent increased intensity in the central region of cells is probably due to image superposition, as the thickness of REFs at this point is 5 μ m and many stress fibre bundles

traverse this region of the cell (56). However, individual stress fibre bundles can be resolved in different planes by differential focusing of the microscope. These still showed a uniform distribution of protein C4.

A contractile role for stress fibres was initially inferred from the periodic distribution of proteins associated with the regulation of contraction in smooth muscle such as tropomyosin, caldesmon, calmodulin and myosin light chain kinase along their length (56,53). More recently, however, contractile activity has been directly demonstrated by the microdissection of stress fibres which then shorten in the presence of Mg^{2+} and ATP (53,56). Furthermore, fibroblasts allowed to adhere to a flexible, silicon rubber substrate will deform (wrinkle) the substrate, which suggests that isometric contraction is occurring (53). The uniform distribution of protein C4 along stress fibres, coupled with its crosslinking activity, suggests that it is not involved in contraction but may *in vivo* align or crosslink microfilaments in the stress fibre bundles to help maintain stress fibre integrity. The appearance of protein C4 concomitant with the formation of stress fibres in cells which have previously been in suspension culture for 72 hours is additional support for this role. A similar functional role has been suggested for actin binding proteins such as α actinin and filamin, which also has a uniform distribution which are thought to crosslink parallel actin filaments into bundles and hold them together (56).

In complete contrast to the high intensity of fluorescence labelling found on actin stress fibres in the subcortical regions of the cell, comparison of phase contrast images, to locate the cell periphery, with immunofluorescence images, it was shown that protein C4 was absent from the highly dynamic and geometrically complex cortical layer of the cell and structures associated with it, such as lamellipodia and membrane ruffles (416,393,417). It is interesting that the thick bundles of actin filaments found more deeply inside the cell are never seen in these areas, except where they terminate at adhesion plaques. The diffuse actin network present deeper in the cytoplasm of fibroblasts (247) was also unstained by anti-protein C4 antibody. It is possible that protein C4 binds preferentially and with higher affinity to α actin isoforms present in stress fibres than to β or γ actin isoforms that are known to predominate in the cortical regions (60) and may explain the distribution of protein C4 inside the cell. The distribution of the high molecular mass tropomyosin

isoforms on stress fibres and not in the cortex, for example, is known to be modified by their affinity for α actin (262).

Although protein C4 is not apparently excluded from areas of cell : cell or cell : substrate attachment, it does not appear to be not enriched at these points where stress fibres associate with the plasma membrane. A number of proteins are enriched at these areas, for example, α actinin, vinculin, paxillin, talin, radixin and integrins and form a transmembrane link between stress fibres and the extracellular matrix or neighbouring cells (269,445). Experiments which follow the incorporation of fluorescently labelled transgelin or protein C4 with time might show whether this molecule is initially incorporated into stress fibres at these points of membrane attachment and then along the filament or if incorporation is more random along the filament length, as was shown for fluorescently labelled caldesmon which was stably and quickly incorporated into stress fibres (490). The precise, quantitative *in vivo* distribution of protein C4 could be defined by using an antibody coupled to an electron dense marker such as colloidal gold, together with thin section electron microscopy.

A possible explanation for these data was that the uniform distribution of protein C4 on stress fibres, as described above, was due to non-specific precipitation of the protein by methanol used for permeabilizing cells prior to immunofluorescence. Additional experiments (a) using cells prefixed with formaldehyde, which crosslinks the lysine residues of proteins thereby immobilising them, (b) microinjection of anti-protein C4 antibody into living cells followed by immunofluorescence (399) and (c) experiments using drugs which interact with different cytoskeletal networks, namely colchicine which disrupts microtubules and intermediate filament networks (245) and cytochalasin D which perturbs the microfilament network (348) substantiated the observations and strongly suggest that the distribution of protein C4 on actin stress fibre bundles is the native distribution of this protein, and is not induced by preparative protocols. These data were further reinforced by immunoblotting of preparations of thin filaments from sheep aorta, which showed the presence of protein C4 on the thin filaments together with several other actin associated proteins such as tropomyosin and caldesmon (285).

3 Distribution of Protein C4 in Various Cell Types.

In cells with well defined stress fibres bundles, intense protein C4 staining was seen specifically along stress fibre bundles. In cells where stress fibres were reduced in number or absent, a more diffuse cytoplasmic staining was seen with anti protein-C4 antibody, although stress fibres that were present were labelled. For example, epithelial cells and transformed fibroblasts have few, thin, vestigial microfilament bundles, and protein C4 staining is seen along these structures but also diffusely in the cytoplasm. The less intense staining of stress fibres in these cells compared to normal fibroblasts may be due to their reduced number leading to less image superposition and therefore a reduced fluorescent signal. Protein C4 also has a diffuse distribution throughout the cytoplasm of motile lymphocytes, which do not contain large F actin bundles, but where much of the actin is associated into a fine cross linked cortex of short microfilaments underlying the plasma membrane (399).

These studies show that cells in which staining with anti-protein C4 antibody is diffuse within the cytoplasm are those in which stress fibres are either reduced in number or absent. In these cells there is a concomitant loss of transgelin expression. It is possible that C4¹, in the absence of transgelin, either binds to G actin or to the meshwork of short filaments of F actin, or is not associated with actin at all. This latter point is unlikely since immunofluorescence experiments have shown that in SV40 transformed fibroblasts (which I have shown express only the lower molecular weight isoform) labelling is still present on the vestigial microfilament bundles present in these cells. To answer this question unequivocally in cells which express either both isoforms, or the lower molecular weight isoform, C4¹, requires antibodies specific to each of the protein C4 isoforms. This can be done with peptides raised against the non homologous regions of these proteins, which cDNA sequence data has revealed (Data included by kind permission of R.K Prinjha and M.A.Smith)

4 Detergent Solubility of Protein C4

The detergent solubility of protein C4 was initially demonstrated when cultures of REFs were detergent extracted in Triton X100 and then examined by immunofluorescence. These observations were extended by using immunoblotting, where examination of the Triton X100 soluble fraction confirms that neither isoform of protein C4 remains associated with the detergent insoluble actin cytoskeleton after detergent extraction. The detergent solubility of protein C4 was exploited in the purification protocol for transgelin, and was further investigated in a permeabilised cell rebinding assay.

5 Protein C4 Polypeptide Doublet

Immunoblot analysis showed that both anti-protein C4 antibodies recognize a closely spaced doublet of molecular mass 21kDa, (as determined by plotting \log_{10} of molecular mass against % mobilities of standard proteins of known mass in SDS PAGE gels). Protein C4 is present in most tissues examined, with expression of either one, or both isoforms. Different tissues can therefore be classified into groups on the basis of their differential expression of the protein C4 doublet

5 i Tissues in which Protein C4 Doublet is Absent

By immunofluorescence and immunoblot analysis, protein C4 expression was undetectable in three cell types, adult skeletal muscle, erythrocytes or neurones, which I will discuss below.

a Adult Skeletal Muscle

Preliminary experiments have shown that protein C4 is present in myoblasts, where diffuse cytoplasmic staining of protein C4 plus staining of actin filaments was found, but is down regulated as myoblasts fuse to form myotubes (399). The changes that occur and lead to the formation of a highly organized skeletal muscle sarcomere are the result of a number of changes in the patterns of contractile protein expression, and involve the repression of non muscle genes and the induction of muscle specific genes. Non muscle β and γ cytoplasmic actin isotypes predominate in replicating myoblasts, but on cell differentiation their synthesis ceases, and the expression of muscle actin isoforms is

induced, with cardiac α actin appearing first and followed by the skeletal α actin isoform. In mature skeletal muscle, the actin present is almost exclusively skeletal α actin, although a band of nonmuscle γ actin similar to the cortical actin array of non muscle cells makes a cytoskeletal bridge between the contractile apparatus and the sarcolemma (180,371,173).

Concomitant with myoblast fusion and the switch in actin isoforms is an increased production of muscle specific isoforms of regulatory and structural actin associated proteins, for example, myosin light and heavy chain isoforms, a muscle specific tropomyosin and troponin which are assembled into the highly ordered sarcomere, while other proteins, such as two tropomyosin isoforms and caldesmon are down regulated on fusion (298).

Protein C4 expression may be down regulated in adult skeletal muscle because its functional role is either fulfilled by another (skeletal muscle specific) protein or its binding is inhibited by a skeletal muscle specific protein, or the paracrystalline structure of the cytoskeleton in myofibrils of adult skeletal muscle coupled with the type of contraction may not require protein C4. Fluorescently labelled caldesmon microinjected into skeletal muscle associates transiently with the A band, possibly due to an interaction with myosin, but it is not stably incorporated into thin filaments. This inhibition of binding may be due to blocking of its association site by the troponin /tropomyosin complex in skeletal muscle, which may take over the regulatory function of caldesmon (490).

b Erythrocytes

Both isoforms of protein C4 are absent in erythrocytes which are highly differentiated and adapted for their specialised role as O_2 and CO_2 carriers. In these cells spectrin is the principal component of the cytoskeleton, and forms a two dimensional network underlying and supporting the plasma membrane, maintaining structural integrity and the biconcave shape. The tail ends of five or six spectrin tetramers are linked together by short actin filaments (15 - 20 monomers) which are supported by tropomyosin to form strikingly regular hexagonal lattice. Band 4.1, adducin and dematin stabilize these junctions, and the spectrin : actin network is linked to the membrane by ankyrin which binds to β spectrin and band 3, a transmembrane protein, and band 4.1 which to binds

glycophorin (163). There are no large microfilament assemblies in these cells and this may explain the absence of protein C4, since I have shown that protein C4 is associated with such filamentous actin structures. It is possible that protein C4 is present in nucleated (immature) erythrocytes from mammals or nucleated erythrocytes from lower vertebrates. This has not yet been examined.

c Neurones

These are highly differentiated cells in which each part of the cell, such as the dendrite, axon, synapse, are specialised either to conduct action potentials, or receive and transmit signals at synapses. Their cytoskeleton is composed of microtubules, neurofilaments, (a tissue specific intermediate filament type), and microfilaments (331). Microtubules are the dominant cytoskeletal element in the axon, where short actin filaments, linked by fodrin, form a network which lies beneath the plasma membrane, suggesting that the actin : fodrin lattice plays a supportive role (156). Actin and actin associated proteins, however, abundant in growth cones, the specialised motile structures found at the tips of elongating neurones. A dense, cross linked meshwork of actin filaments together with myosin, α actinin, tropomyosin and fodrin, is found behind the ruffling margins, filling the flattened palm of the growth cone, whereas bundles of actin filaments, crosslinked by fimbrin and emerging from this cortical cytoskeleton fill the actively extending and retracting microspikes (156,242). In neuronal development, growth cones lead extending axons towards their distant target sites by pathfinding, and reorganization of the growth cone actin cytoskeleton in response to environmental cues underlies axon guidance (117,36). The growth cone is the developmental precursor of presynaptic terminals, and the actin cytoskeleton may play a role in synaptic function, by modulating the release of neurotransmitters (117). On the post synaptic side, dendritic spikes that extend from the cell body contain core bundles of filamentous actin (117). The parallel actin arrays with which protein C4 associates are not found in these cells and may explain its absence. Alternatively, an additional molecule may well fulfill the role of protein C4. It is interesting that although protein C4 is absent in neurones, our cDNA data has revealed the presence of a highly homologous molecule, NP25, in these cells (357). It is

possible that this protein may bind and cross link the linear actin filament bundles which are known to be present in, for example, the microspikes of growth cones.

ii Tissues in which only the Lower Molecular Mass Isoform, C4^l is Present

Only the lower molecular mass isoform of protein C4 was found in lymphocytes (399), heart, epithelial cells and transformed mesenchymal cells. By immunofluorescence epithelial cells and transformed cells do not have the large microfilament bundles that are such a feature in normal mesenchymal and smooth muscle cells. Cells which express only C4^l show cytoplasmic protein C4 staining and the antibodies also stain the vestigial actin bundles which are present in these cells.

Cardiac tissue, in which transgelin is absent, expresses a 79kDa isoform. The expression of C4^l and the absence of transgelin may reflect the actin isoform (α cardiac) that is found in this tissue (180,371). Detection of C4^l in brain tissue is surprising, given the absence of both isoforms of protein C4 in neurones. It is possible that this isoform is expressed by other components of the brain tissue, such as glial cells, endothelial cells, and circulating lymphocytes, although this has not yet been investigated.

The function of C4^l has not yet been determined and it is not yet known why the expression level of this protein varies in cells such as normal fibroblasts and smooth muscle. That both isoforms are found on actin suggests that they may be related (at least in part) functionally, possibly in a regulatory cascade. For example, C4^l may modulate transgelin's actin binding activity. To determine the function of C4^l will require purification of this isoform, which can then be used in functional assays.

iii Tissues in which the Protein C4 Doublet is Present

Although both protein C4 isoforms were found in smooth muscle, a preponderance of the higher molecular mass isoform was seen in this tissue, while normal mesenchymal cells expressed both isoforms in approximately equal (but variable) amounts. These data suggest that the organization of actin filaments in stress fibre bundles in fibroblasts and smooth muscle (in the absence of skeletal muscle specific isoforms of associated proteins) requires the presence of the higher molecular mass isoform, transgelin.

Smooth muscle is the least differentiated muscle type, and lacks the paracrystalline structure of the actin and myosin myofibrils found in skeletal muscle sarcomeres (268). It is composed of sheets of elongate spindle shaped cells that contain thick filaments of aggregated myosin and thin filaments of actin and associated in a loosely arranged contractile apparatus (465). They are roughly aligned with the long axis of the cell and attach obliquely to the plasma membrane at disc like junctions that connect groups of cell together. This orientation permits a high degree of cell shortening and therefore produces large movements even without the leverage provided by attachment to bone (465). Contraction of smooth muscle is slow, and sustained. This long term maintenance of tension may be facilitated by the reversible crosslinking of actin by, for example, caldesmon, and it is possible that protein C4 or transgelin has a similar function in maintaining the latch-bridge state. Alternatively, it may maintain thin filament integrity, by cross linking individual actin filaments together within a thin filament

Protein C4 expression may be developmentally regulated in aorta, in parallel with actin isoforms and actin associated proteins. For example, smooth muscle specific isoforms of actin (α smooth), myosin light and heavy chains, caldesmon, calponin and metavinculin are present in mature smooth muscle cell (146,184,98)

The eight isoforms of actin are expressed in a tissue specific manner that is conserved across species, implying that their presence is functionally important. Quantitative differences in their interactions with associated proteins may modulate the functions of actin in the cell(371). Protein C4 is absent in skeletal muscle, (possibly because the highly ordered arrangement of the contractile apparatus is maintained by myosin : actin cross bridges and the cross linking activity of skeletal muscle specific proteins, rendering protein C4 unnecessary), present in cardiac muscle (C4^l) and present in myoblasts, vascular and enteric smooth muscle and mesenchymal cells (both isoforms). Although these cells all generate contractile force by means of actin : myosin interactions their actins differ in (a) amino acid sequence (454) (b) spatial arrangement and (c) association with different sets of actin associated proteins (286). The contractile apparatus of skeletal muscle contains α skeletal actin, with non muscle actin isoforms restricted to sub sarcolemmal regions, while cardiac muscle contains α cardiac actin. Stress fibres in aortic smooth muscle and

fibroblasts are composed of α smooth muscle actins, with β and γ non muscle actins found in the cortical regions of these cells (180,371). Protein C4's presence on stress fibres and absence from the cortex of fibroblasts, and its presence in smooth muscle (immunofluorescence experiments on sections of smooth muscle are required to determine its precise location, although it is associated with thin filaments purified from aorta) might indicate a higher affinity of protein C4 for α smooth muscle actin, and that it is expressed in these tissue types concomitant with smooth muscle α actins. The expression of α smooth actin may regulate protein C4 expression, as, for example, has been shown for tropomyosin isoform expression which is influenced by the ratio of β : γ actin.(386). It is possible that protein C4/transgelin has a role in the maintenance of sustained contractions that are a feature of the contractile apparatus in these cells (465,56) Its exclusion from other muscle actins may be due to competition for binding, or steric hindrance of binding by other proteins that associate preferentially with α skeletal muscle, and β and γ non muscle actins. Transgelin is able to bind to α skeletal muscle actin *in vitro*, as shown in the functional studies, where actin purified from rabbit skeletal muscle was used.

iv Protein C4 Doublet Formation

The formation of the proteinC4 polypeptide doublet could be the result of several factors:

(a) Proteolytic cleavage of transgelin, the higher molecular mass isoform, to produce C4¹ during sample preparation. However, proteolysis products are usually seen as a ladder of distinct bands, coordinately reduced in size and intensity, whereas C4¹ appears as a single discrete band. By using a spectrum of protease inhibitors the majority of known lysosomal proteases, the major source of cell proteolysis were inhibited. Those that could not be inhibited, the carboxypeptidases, have a pH optimum of 2.5 - 5.5 and so are less likely to function at a pH of 7 (358), the conditions of sample preparation. It seems unlikely, therefore, that C4¹ is a proteolytic cleavage product of transgelin.

(b) The appearance of the two component polypeptides, even in non reduced SDS PAGE gels (thus preserving intermolecular disulphide bonds) suggests that the doublet is not a disulphide bond linked dimer (399).

(c) Since partially reduced intramolecular disulphide bonds would cause the non reduced species to have a more compact structure and higher electrophoretic mobility, as is known for the integrins (199), to ensure that any internal disulphide bridges in protein C4 were fully reduced, experiments were carried out in 100mM DTT or 20% 2ME, which are both reducing agents. No differences in the mobilities of the protein C4 bands were observed. Recent cDNA studies have further substantiated these data and have shown that there is only one cysteine residue in the sequence of transgelin, therefore it could not form intramolecular disulphide bonds (357).

(d) That C4¹ is modified or cleaved from transgelin, as a post translational modification, with the modification proceeding faster in some cells such as epithelial cells and transformed fibroblasts to generate a single detectable band is militated against by *in vivo* metabolic labelling/immunoprecipitation experiments which show that there is no precursor/product relationship between transgelin and C4¹. Since methionine residues may have been reduced in number or absent in either of the two isoforms, I used both [³H] leucine and lysine and [³⁵S] methionine in immunoprecipitation experiments with no detectable difference in the results. These showed that after a 4 hour pulse transgelin incorporated 2-4 fold as much radioactivity as C4¹. This suggests that either (a) transgelin has an increased number of leucine and lysine, and methionine residues (b) transgelin synthesis occurs at a greater rate, due possibly to greater instability (c) transgelin is preferentially immunoprecipitated by anti-protein C4 monoclonal antibody, reflecting a reduced number of epitopes recognised by the monoclonal antibody in the lower band, so that it is less efficiently immunoprecipitated or (d) the amounts of the two isoforms present in the cells varies. No reduction in this ratio over a 48 hour chase period suggests that the longer term turnover and degradation of the epitope in both polypeptides is equal. Furthermore, the lack of any apparent increase in radioactivity present in C4¹ and concomitant decrease in transgelin over the chase period strongly suggests that there is no precursor/ product relationship.

It is likely therefore that transgelin and C4¹ exist as a non covalently linked dimer, or as monomers, to determine this will require analysis of the interaction between the two

purified proteins in, for example, linear sucrose gradients (288) or equilibrium column chromatography (189).

Furthermore, recent cDNA data shows that transgelin and C4¹ are the products of two separate genes, whose expression may be regulated independently. (Data included by kind permission of R K Prinjha and M.A.Smith)

6 Expression of a 79 kDa Isoform

In addition to C4¹, immunoblot analysis of heart tissue showed an additional band at 79kDa in heart tissue. Preliminary studies demonstrated that this isoform may be associated with the stress fibres that are found in endothelial cells of the arterial vascular system. Stress fibres in these cells are oriented parallel to the direction of blood flow, and are prominent in endothelial cells from regions of vasculature exposed to high velocity flow and turbulence, such as the left ventricle and atrium, together with aortic valve and aorta. Their role is thought to involve helping endothelial cells withstand haemodynamic stress, protecting against haemodynamic injury and detachment (56,488). Stress fibre formation can be induced in endothelial cells cultured *in vitro* by exposing them to levels of fluid shear stress equivalent to that occurring in human arteries. Stress fibres in these cultured cells will realign in the direction of blood flow (120) and interestingly, there have been indications that the concentration of actin increases in endothelial cells in hypertensive animals (56) It is possible that the expression of the 79kDa protein is involved in the cytoskeletal remodelling of stress fibres that is known to occur when endothelial cells are exposed to mechanical stress *in vitro* or *in vivo* in cells from regions of high haemodynamic stress. Furthermore, it would be interesting to see if expression of the 79kD isoform could be induced in cells placed under mechanical stress (229) or fluid stress which can be done by using rheological techniques (120). It has been shown that in epithelial cells held under tension by micromanipulation with a needle all lateral cytoplasmic protrusions are withdrawn as the microfilaments align parallel to the direction of the applied tension (229). Cells in areas of particularly high stress which have a greater need to adhere might recruit additional proteins, such as 79kDa to maintain tension in the stress fibres. In scleroblasts, stress fibres are especially prominent in cells near the edges of the scale or

over radiating ridges, reflecting the greater need for cells to adhere to the substrate in these regions of increased shear forces (56). Investigation of such cells could be carried out to see if they contain this particular isoform. The presence of 79 kDa in such cells may offer clues as to its cellular role, but future work in this area will initially involve purification of this protein and studying its function by using the panel of *in vitro* assays with which I am now familiar.

7 Evolutionary Conservation of Protein C4

All eukaryotic species contain actin and the genes coding for this protein are highly conserved in evolution, with, for example, yeast and human actins having 89% homology (396). This high degree of conservation may be the result of the requirement of actin to interact with many different proteins to achieve its full organizational repertoire (426).

In addition to actin, actin associated proteins, for example, vinculin and tropomyosin (16, 68,115) are highly conserved during evolution. Comparisons of the deduced amino acid sequence of other cytoskeletal proteins, from *Dictyostelium discoides*, in particular α actinin and severin, show striking similarities at distinct regions with proteins from higher organisms including mammals. It is likely therefore that regions that are structurally or functionally important, such as the EF hand motif and actin binding domains are maintained through evolution (387).

The inherent conservation of cytoskeletal proteins led to an investigation of the evolutionary conservation of protein C4. Studies with monoclonal antibody anti protein C4 have shown that the molecule is present in most tissues in all vertebrate species so far examined, including human, sheep, mouse, rat, chick, frog and fish. The epitope recognised by this monoclonal antibody is also present in organisms phylogenetically well removed from vertebrates, such as crustaceans and molluscs, but here the molecular masses are 57 and 18 kD respectively (399).

Since a monoclonal antibody recognizes only one epitope of usually 5-7 amino acids (169), I raised and affinity purified (using purified protein C4 coupled to beads) a rabbit polyclonal antibody against protein C4 has been shown by immunoblot analysis to recognize the polypeptide doublet.

By using the broad spectrum polyclonal antibody I extended my preliminary observations using monoclonal anti-protein C4 antibody and found that transgeline was also present in the fission yeast, *Schizosaccharomyces pombe*, where it has the same apparent molecular mass in SDS PAGE as transgeline from higher eukaryotic cells. There was no evidence of the C4¹ isoform of protein C4. Yeast are known to have one gene that encodes actin, and recently a gene that encodes for a distantly related member of the actin protein family, ACT2, has been isolated from *S.pombe* (257). Actin structures such as dots at the growing end of the cells, and a filamentous band at the equator, which appears to dictate the position of septum formation at cell division, and fine filaments of actin have been observed in fission yeast. The distribution of actin in the cell, which is associated with deposition of cell wall components and cell wall growth, changes through the cell cycle, and is necessary for proper functioning of the cell. The changes in distribution are not accompanied by a significant increase in actin synthesis and must therefore be due to the reorganization of existing actin either by depolymerisation and repolymerisation or redistribution of actin filaments (211,282) and it is highly likely that actin associated proteins, such as ACT 2 which is thought to act as a capping protein, profilin, tropomyosin, myosin (96) and protein C4 found in *S.pombe* regulate the reorganization of the actin cytoskeleton. It would be interesting to carry out genetic manipulations to try to determine the *in vivo* function of transgeline in yeast.

8 Comparisons of Protein C4 with Other Actin Associated Proteins

Immunoblot analysis of samples of known actin binding proteins of similar mass to protein C4, including chick light chain myosin (399), bovine calmodulin 399), porcine cofilin (318), chicken brain actin depolymerizing factor (11), and N-ras p21 (447) has indicated that protein C4 was a novel protein.

However, to extend this work at the level of the gene, and determine the function of transgeline, I purified transgeline. It was amino acid sequenced in collaboration with Dr Justin Hsuan and Dr Nick Totty at the Ludwig Institute and gave 122 amino acids of sequence, including the amino terminus, representing ~ 60 % of the complete protein.

Analysis of the amino acid and cDNA sequence of transgelin in data bases has revealed that transgelin shares homology with a 22kDa protein, SM 22 α (83.6% homology) which is found mainly in smooth muscle and has an Mr and pI similar to transgelin, although no interaction with F actin nor with actin associated proteins has been demonstrated. Similar homology has been found with WS3-10, a protein present in fibroblasts which may represent the human homologue of SM22a, and p27, a protein which associates with the actin cytoskeleton of fibroblasts. No function has been ascribed to these proteins. In addition, homology was found with calponin, the product of the *Drosophila* gene mp20, and NP25 a neuronal protein. (357, 400)

9 The Effect of Viral Transformation on Protein C4

I have studied the effects of oncogenic transformation by Simian virus 40(SV40), a DNA virus, and Rous Sarcoma virus (RSV), an RNA retrovirus on protein C4/transgelin by comparing its expression in cells transformed by these viruses with their normal counterparts.

While the two types of viruses act differently [via small t and large T in the case of SV40 (107) and pp60^{v-src} in the case of RSV (217)] cells transformed by either show similar changes in their phenotypes, including changes in cell morphology, biochemistry, behaviour and growth characteristics.

Transformation is often accompanied by a striking change in the actin cytoskeleton, with a large scale disruption of microfilament bundles, as actin fails to organise into stress fibres and is distributed diffusely through the cytoplasm or concentrated in a meshwork beneath the cell surface (251,111). Associated with this reorganization of actin filaments are excessive blebbing of the plasma membrane (86), increased ruffling of the membrane (313), the increased mobility of plasma membrane glycoproteins (89), a marked decrease in the number of adhesion plaques and change in their structure with the formation of invadopodia (91,59) altered adhesion to the substrate and other cells, a rounded morphology with few well defined cell processes, and the increased mobility of transformed cells (137).

Biochemically these cell shape changes may be associated with down regulation of expression of the α actin isoform that is abundant in normal fibroblasts and is associated with the flattened, well spread morphology of normal cells (252), disturbances in the balance of actin isoforms (253), and the expression of variant forms of actin which may be polymerisation incompetent (456,251). In addition to the changes in microfilaments that may be brought about by qualitative changes in actin expression, the reorganization of actin which is an important aspect of transformation may be the result of alterations in the expression of actin associated proteins that control actin filament assembly and structure. The expression of several actin associated proteins is known to be down regulated in transformed cells, including high Mr isoforms of tropomyosin (294), non-muscle caldesmon (337), smooth muscle myosin light chain 2 (234), the low Mr isoform of calponin (98), thymosin β 4 (491), gelsolin (457), ABP 280 (86), MARCKS (215), α actinin (145), vinculin (113), E-cadherin (463), α catenin (441) and $\alpha_5\beta_1$ integrin (345) .

This study has shown that transgelin is also down regulated in transformed cells. Normal rat (Rat 1) or mouse (3T3) fibroblasts express both polypeptides of protein C4. However, on transformation of Rat -1 cells with RSV or 3T3 cells with SV40 only C4¹ is expressed, while transgelin is completely down regulated. Transformation by distinct oncogenic viruses, DNA (SV40) and RNA (RSV), therefore produce a common effect on protein C4 expression. These data have been reinforced by recent work at the level of the gene which show that in transformed cells the message encoding transgelin is absent (357).

The observations that transgelin is absent in transformed cells and epithelial cells which have few if any stress fibres, and in motile lymphocytes which do not have actin filament bundles (399), while it is abundant in mesenchymal cells and smooth muscle which have prominent stress fibre arrays suggests a correlation between transgelin expression and the presence of well defined stress fibres. Given transgelin's crosslinking role, inducing gelation of actin filaments *in vitro*, and its association with stress fibres, it is possible that its role is to control or maintain stress fibre organization and integrity, and that the down regulation of this protein in transformed cells may, in part, be responsible for the loss of organised stress fibres that, together with enhanced motility, are such a feature of

these cells. Transgelin may be responsible in part for maintenance of a normal phenotype in non transformed mesenchymal cells. It would be interesting to transfect cDNA encoding transgelin into transformed cells, to see if its expression could suppress the transformed phenotype.

Some or all of the actin associated proteins, mentioned above (including transgelin) may act synergistically to organise and control actin architecture in cells, and alterations in their expression may therefore be directly or indirectly involved with acquisition of the transformed state (457). Changes in the level of caldesmon (which enhances tropomyosin binding), increased expression of calcium and calmodulin and a 55kDa actin binding protein which inhibits the actin binding of low Mr tropomyosins, coupled with replacement of high Mr with low Mr tropomyosins in transformed cells are likely to decrease the binding of tropomyosin to actin filaments (492), and because this tropomyosin binding stabilizes the structure of actin filaments (by protecting actin filaments from the activities of fragmin, villin, DNase I and ADF [262]) this may result in the destabilization of stress fibres in transformed cells and cause, in part, the morphological alterations observed in transformed cells. Modifications in actin expression, and disturbances in the balance of isoforms, to which actin associated proteins bind with altered affinities (386) may also be associated with actin cytoskeleton rearrangement and alterations in cell shape in transformation.

The reason that the changes in microfilament organization on transformation appears to involve the coordinated down regulation of several elements of a structural and functional actin organization, rather than a single protein (457), may reflect the redundancy of these molecules and the ability of one actin-associated protein to substitute for another, although the expression of a single protein, for example, tropomyosin (355), α actinin (145) or vinculin (113) can suppress the tumorigenic phenotype. A fully transformed phenotype may require changes in a range of proteins. It is unclear at present how transgelin interacts with other actin associated proteins and therefore how it might be involved in the cascade of events that lead to the transformed phenotype. I plan to investigate, using the techniques of equilibrium column chromatography (189), linear sucrose gradients (288), and *in vitro* functional assays the interaction of transgelin with

other actin associated proteins. I will initially look for interactions between transgelin and C4¹.

10 Suspension Culture and Control of Protein Synthesis

Some of the events associated with transformation, for example, cell shape change, reduced substrate adherence and cytoskeletal alterations can be induced in normal anchorage dependent cells by culturing them in suspension. Cells in suspension culture are blocked in G1 stage of the cell cycle, and DNA, RNA and protein synthesis are inhibited (22). The decline in the synthesis of most proteins is gradual and extensive, so that after 72 hours in suspension culture protein synthesis amounts to only 10-15% of the expected rate for control cells growing in a monolayer (17). Vimentin is gradually down regulated, while the synthesis of actin relative to other proteins is specifically inhibited (25). However, normally attached fibroblasts had to be suspension cultured for 72 hours before transgelin expression was completely down regulated, and transgelin was not detected. In every instance, the expression of the lower molecular weight isoform C4¹ was unaltered.

In most cases which have been studied the decline in protein synthesis is not due to generalised cell damage, but is the result of a reversible modification to mRNA which is stabilized in a non translatable form (17,108). Although mRNA production is down regulated five fold in suspension cultured cells, the level of mRNA remains constant since the majority of mRNAs are stabilized against degradation, and acquire an extended half life (108).

On reattachment of cells to a substrate, there is a general rapid recovery of protein synthesis of up to 6-7 fold 4 hours after attachment (23). This occurs primarily at the level of translation as preexisting mRNA is reactivated and used for protein synthesis (17,108). For example, the gradual down-regulation of vimentin is quickly reversed to control levels after about 6 hours in adherent culture (25). In contrast, transgelin reexpression is not detected until 24 hours after reattachment, suggesting that the mRNA coding for this protein is not stabilized against degradation and therefore is absent in suspension cultured cells. While the mechanism by which this occurs is unknown at present, it is possible that transgelin mRNA stability is controlled in a similar fashion to tubulin where the depolymerization of microtubules leads to a rapid reduction in the rate of tubulin synthesis in parallel with a reduction in the translation

activity of tubulin mRNA. Tubulin auto regulation is a cytoplasmic event which involves the recognition of the first 4 nascent N terminal amino acids of tubulin on polysomes by unpolymerised tubulin subunits. Protein:protein interactions regulate activation of an RNase which degrades tubulin mRNA (25,71). It is possible that as transgelin detaches from disassembling stress fibres it recognises nascent transgelin polypeptides and initiates degradation of its mRNA. Unlike transgelin which is degraded, C4^l is present in suspension cultured cells, and it is possible that the mRNA that encodes it is stabilized.

While most proteins recover synthesis coordinately, 8 hours after replating when protein synthesis has recovered to control levels, actin synthesis is maximal and accounts for 25% of total protein synthesis. This requires the new synthesis of mRNA in addition to pre-existing mRNA (108,17). Transgelin, however, could not be detected 8 hours after replating. In this study, cells that had been returned to adherent culture for 8 hours were well spread, but at this time F actin was not organized into stress fibre bundles. This did not occur until 24 hours after reattachment, a time when levels of transgelin expression were indistinguishable from control cells, supporting the observation that, *in vivo*, transgelin may be involved in the formation and initial stabilization of actin stress fibre bundles.

Small, subtle changes in spreading have a large effect on DNA synthesis and while recovery of protein synthesis within a few hours of replating is triggered by the establishment of only a few contact points between the cell membrane and the substrate, recovery of metabolism associated with the nucleus, DNA rRNA and mRNA synthesis, is much slower and requires extensive cell spreading (23). Furthermore, alterations of microtubule and microfilament organization may dramatically affect the initiation of DNA synthesis, suggesting a role for these networks in transducing signals exerted at the cell surface by growth factors to bring about initiation of DNA synthesis. In suspension cultured cells the structural networks that normally transduce cell shape/ surface contact signals to cell metabolism are disorganized (25). Cells in suspension culture are blocked in G₁, and on reattachment and subsequent spreading they proceed out of G₁ and enter subsequent stages of the cell cycle. DNA synthesis starts 14 hours after replating and peaks 20 hours after reattachment (23,22) Our finding that 24 hours after replating are necessary for the reexpression of transgelin suggests that expression is likely to be under transcriptional rather than translational control. This was

confirmed by incubating cells for the initial 24 hour attachment period in either cycloheximide (which blocks de novo protein synthesis [83]) or actinomycin D (which blocks mRNA production [172]) at concentrations which do not effect viability. Both these drugs completely blocked transgelin expression.

Expression of transgelin is not simply related to cell shape change since after 8 hours reattachment when cells are spread no transgelin expression is detectable. It is possible therefore that the induction of transgelin is associated with some other cellular event. This may well involve the formation of stress fibres since acquisition of transgelin is concomitant with formation of stress fbres in fibroblasts respreading after 24 hours. Furthermore, the absence of transgelin is associated with (a) reduction in stress fibre numbers in mesenchymal cell transformation (b) acquisition of a rounded morphology and concomitant loss of stress fibres in mesenchymal cells that normally have a full complement of them (c) absence or reduced numbers of stress fibres in cells such as lymphocytes and epithelial cells and (d) geometric arrays of actin in the cortex.

11 Protein Purification

11 i Purification of Transgelin from Sheep Aorta

The down regulation of transgelin expression in transformed mesenchymal cells, and in normal mesenchymal cells when they are maintained in suspension culture, coupled with its absence in non-adherent cells such as lymphocytes and cells with a rounded morphology where stress fibres are reduced in number or absent such as epithelial cells dictated that I purify transgelin and study its characteristics, function and interactions with actin.

Although the anti C4 antibody affinity column I used initially was successful for purifying small amounts of protein C4, its use was limited because (a) the protein was eluted from the column with a high pH buffer (pH 11.5) which may result in some unavoidable denaturation of the protein and therefore affect the structural integrity and activity of the molecule, and (b) the immobilized ligand, monoclonal anti-protein C4 antibody recognizes only one epitope. This monovalent binding is therefore weak, and may result in a loss of protein C4 due to leaching from the column. To bypass these problems I

tried several alternative methods for the purification of transgelin in its native configuration before finalising on the protocol that is now used routinely, as described in methods section.

Small pieces of frozen aorta were homogenized and extracted in a large volume of buffer, to minimize the percentage loss of detergent soluble protein in the pellet. As tissue is ruptured in the homogenization step, many proteases and especially lysozymes are released, and therefore protease inhibitors were included in the extraction buffer, and the buffer and all equipment were chilled to 4°C (358). Although the majority of proteases are removed during initial purification steps, trace amounts can still cause considerable proteolysis, therefore a range of protease inhibitors to provide broad spectrum protection against proteolytic degradation were included in buffers at all stages of purification (358). Mechanical disruption of tissue with a Waring Blender can cause local overheating and denaturation, and to prevent this the blender was cooled and several short bursts, with the vessel being cooled in ice water in between, was used in preference to one long burst. This also helped to minimize foaming and shearing, further reducing protein denaturation (358). CHAPS, a zwitterionic detergent, was used to extract transgelin. CHAPS has the following advantages over a non ionic detergent such as Triton X100: (a) a high critical micelle concentration (the minimum concentration at which detergent monomers begin to form micelles) and a low micellar molecular weight (therefore it can form small mixed micelles), it can therefore be removed easily by dialysis (b) is non-denaturing (c) disperses protein aggregates at concentrations of up to 0.5% w/v (d) does not absorb at OD 280nm and (e) it does not hide charge groups and thereby alter the binding behaviour of the protein on charge columns (81). On disruption of the cell membrane and release into the buffer, proteins encounter an oxidizing environment, which can cause inactivation or denaturation and aggregation. To militate against this I used DTT, a reducing agent in the buffer (358)

Most of the actin and tropomyosin was removed from the preparation by centrifugation after a high salt (0.5M KCl), pH 4.8 dialysis step. The solubility of proteins varies with pH and is least near their isoelectric point. At their pI the negative and positive charges on the surface of the molecule cancel each other out, electrostatic repulsion between individual molecules no longer occurs, electrostatic attraction increases, resulting in

individual proteins aggregating and then precipitating. Unwanted proteins can be removed in this way (358) In this case actin was precipitated out of solution at pH 4.8, which is close to its pI of 5.2.(501). A protein that was probably tropomyosin from its apparent molecular weight was also removed, possibly because of its association with actin, and pI of pH4.2 (115).

The next step in this protocol utilized a chromatofocusing column which separates proteins according to their isoelectric point, with high resolution.(358,344) Possible contamination of the transgelin preparation with the more acidic, lower molecular mass protein C4 isoform, C4¹ was avoided by (a) purifying transgelin from aorta where it is the major isoform present and (b) using chromatofocusing, since the two isoforms have different isoelectric points .

The resolution of the column was maximised by using a narrow pH gradient, pH 9.4-7, with the pI of transgelin (pH 8) in the middle, so that it elutes after ~ one third of the pH range. PolyBuffer, diluted to the same buffering capacity as the start buffer (25mM ethanolamine), was adjusted to pH7 and used to elute proteins from the ion exchanger initially equilibrated to pH 9.4 with start buffer. As the PolyBuffer runs through the column, a pH gradient is formed automatically on the column, by virtue of the buffering action of the charged groups of the ion exchanger. The charge on a protein is dependent on the pH and the isoelectric point of the protein. When the pH of the column is less than the pI of a protein, the protein carries a net positive charge and migrates down the column of anion exchanger in the eluting buffer until the pH of the buffer surrounding the protein is greater than its pI. The charge on the protein reverses and it binds to the column. It remains bound until the developing gradient causes the pH to drop below its pI, when it is released from the matrix. The protein is carried in the buffer again until the pH rises above its pI and it rebinds. This process is repeated until the protein emerges from the column at its pI. Proteins with different isoelectric points migrate different distances on the column before binding, and elution is in order of their isoelectric points. Low buffer concentrations of equal buffering capacities which create a linear, fairly flat pH gradient and gentle pH changes, and the use of a long narrow column increase the resolution of the chromatofocusing column. These columns allow a large sample volume to be applied, the

volume is unimportant provided all the sample has been applied before the protein of interest is eluted. This is an advantage over size columns where only a small volume can be applied for maximum resolution (358,344). One of the main contaminants of the aorta preparation before chromatofocusing is a protein of apparent molecular mass 69kD. By use of this column, most of this contaminant is removed by retention on the column, while transgelin is eluted at pH8, in agreement with its pI calculated from NEPHGE gels.

Chromatofocusing was followed by an hydroxylapatite (crystalline calcium phosphate) column. Protein : hydroxylapatite interactions are a function of the net charge on the protein and occurs by both non-specific electrostatic attraction between protein positive charges and phosphate groups of hydroxylapatite and by specific complexing of protein carboxyls with calcium loci on the mineral (153). It has been suggested that acidic and neutral proteins bind to the hydroxylapatite calcium while basic proteins adsorb to the surface phosphate groups. Elution occurs either as a result of non specific screening of charges or by specific displacement of protein groups from sites on the column with which they had complexed (153,154,155). A basic protein such as transgelin would bind to hydroxylapatite by general electrostatic interactions between amino groups and the general negative charge on the column, and elution is most likely to be by charge screening. Interestingly, only native proteins will bind to hydroxylapatite effectively, which indicates that transgelin is non denatured.

Since hydroxylapatite adsorbs proteins by a different mechanism to other separation techniques it is useful for resolution of proteins not achievable by other methods. Substances such as EDTA with a stronger affinity for calcium than phosphate reduce capacity of hydroxylapatite and must be excluded from buffer. Buffer at pH 6.8 was used since hydroxylapatite's capacity for proteins is highest near to neutrality. Potassium, rather than sodium, phosphate buffer was used because of its increased solubility at 4°C (358). Batch elution at 100mM K phosphate eluted transgelin in a sharp peak, removed trace amounts of contaminating proteins, separated transgelin from Polybuffer and reduced the need for significant further concentration. Immunoblot analysis showed that transgelin was the only protein present in the sample, and that it was not apparently degraded. This was

confirmed by reverse phase chromatography (in collaboration with J HSuan and N Totty[400]) which indicated the presence of a single protein peak.

By the protocol discussed above I rapidly purified a high yield of transgelin in its native state as defined by its capacity to bind to purified actin in a saturable manner *in vitro*, to gel polymerized actin *in vitro* and to bind to actin in a permeabilized cell system.

11 ii Actin Purification

Experiments investigating actin polymerization and network formation/gelation require actin that is completely free of trace contaminants such as myosin, tropomyosin and other factors that are known to alter the properties of actin assembly and disassembly (339,274). Conventional preparations of actin, even following cycles of polymerization and depolymerization, may contain ribonucleotides, polysaccharides or inhibitors of polymerization which cannot be detected by Coomassie staining of SDS PAGE gels (339). Gel filtration was used as an additional purification step here, to remove these contaminants, together with actin oligomers and aggregates of denatured actin (which elute in the void volume of the column) (339, 163).

In addition, by separating out a fraction of minor contaminants which block actin filament network formation (by binding to actin filaments) and thus reduce viscosity, without affecting polymerization, column purification significantly increases the low shear viscosity of actin (see section) compared with actin which has not been gel filtered. These potent inhibitors of actin filaments constitute considerably less than 1% of protein in a conventional preparation (274,339). Gross overloading of actin prepared by column chromatography on SDS PAGE gels showed that there were no contaminants, and this was confirmed by an increase in the apparent low shear viscosity from 5s/cm for conventionally prepared actin to 30s/cm after gel filtration.

12 Investigation of Transgelin Function

The techniques used for many years now as standard methods for investigating *in vitro* actin : actin associated protein interactions include low shear viscometry, pyrene actin

fluorescence assays, light scattering assays and electron microscopy, which I have used to investigate the *in vitro* function of transgelin.

Falling ball viscometry is a well documented technique used to measure the rapid changes in apparent viscosity that occur as actin monomers polymerise into filaments and these filaments form networks, or gel, under the influence of ionic strength, cations and a variety of actin associated proteins (352). In this study, falling ball viscometry has been used to analyse the gelation of actin filaments by transgelin.

Pyrene labelled actin is used to measure actin polymerisation (79,52). In this study, it has been used to study the effect of transgelin on the nucleation and elongation of actin filaments.

Light scattering assays have been used to measure actin polymerisation and the crosslinking/bundling activities of actin associated proteins (78). In this study it was used to investigate the crosslinking role of transgelin.

Negative stain electron microscopy (311,212) allows the direct observation and visualisation of the transgelin /actin filament complex *in vitro.*, and the effect of transgelin on actin filaments was clearly seen.

The use of falling ball viscometry showed that transgelin interacts directly with actin, as they are the only two proteins present in this system, and induces actin gelation within two minutes, while in the absence of transgelin, although an increase in viscosity was seen as actin monomers polymerised into filaments, gelation did not occur. This immediately ruled out any depolymerising, severing or capping activity for transgelin, since this would have reduced viscosity of the actin solution as has been shown for actophorin (273) or gelsolin (496) and is in agreement with the distribution of transgelin along intact stress fibres in cells. It also indicates that it does not stabilize actin filaments in a manner similar to tropomyosin which by binding to the sides of actin filaments increases their rigidity, making them less flexible and decreasing the extent to which the filaments can entangle, thereby reducing the viscosity (290).

In the initial stages of this investigation, it was possible that transgelin might have increased actin viscosity and induced gelation by acting at any of the stages in actin network

formation. The gelation of actin requires the polymerization of G actin monomers into polymers, and these polymers must be crosslinked into a network. Actin polymerization from monomers proceeds in 4 reversible steps (a) activation (b) nucleation (c) elongation and (d) annealing (351).

Actin filaments in solution form a large, three dimensional network of flexible polymers and exhibit a high viscosity at low shear rates due to the entanglement and self associations of filaments (352). The degree to which these associations form and therefore alter viscosity is affected by the length of the filaments and their orientation, and any factor that alters the length of the filaments, stability or orientation of cross links will therefore affect viscosity. Addition of a cross linker to actin filaments causes them to form a gel (in which the filaments are crosslinked into an infinite continuous network) either by stabilizing existing filament : filament associations (163) or by forming cross-bridges between individual filaments (327,416).

A role for transgelin as a potent nucleator of actin polymerisation was ruled out by low shear viscometry experiments which investigated the time course of transgelin-induced actin gelation, in which it was shown that an initial lag phase of between 1-2 minutes occurred in both the presence and absence of transgelin, after which time the apparent viscosity of actin alone increased to 30s/cm and remained unchanged after 30 minutes. In the presence of transgelin, the lag phase was followed by a (sharp) increase in viscosity, to the gelation point. Preincubation of transgelin with G actin monomers prior to the initiation of polymerization did not affect the time course of gelation/polymerisation, whereas a nucleating protein might be expected to decrease the lag phase after a period of incubation with actin monomers, by inducing their association into oligomers which act as seeds for polymer growth. No nucleating activity seen in experiments where the incorporation of actin monomers into filaments was measured by an increase in the fluorescence of pyrene labelled actin upon polymerization. By following the increase in fluorescence of actin samples in the absence and presence of transgelin in polymerising conditions it was shown that transgelin did not reduce the lag phase, but that in both cases the time profile of polymerisation was identical. Nor was there any indication of nucleating activity in the light scattering assay. These experiments also suggest that transgelin has no activity as a

monomer sequestering protein (such as thymosin β 4 [374]), thus preventing polymerization, nor to disaggregate spontaneously formed actin oligomers (such as actobindin [240,415]), as these activities would have resulted in an extension of the initial lag phase and possibly a diminished increase in viscosity.

Since apparent viscosity depends strongly on filament length (416), another possibility was that transgelin increased actin filament elongation, by enhancing the addition of monomers to preexisting actin filaments. This was discounted by measuring the incorporation of pyrene labelled actin monomers into actin filaments. F actin filaments were prepared in the presence and absence of transgelin, and then actin monomers, of which 20% were labelled with pyrene, were added. Transgelin neither enhanced, nor reduced the observed increase in fluorescence, indicating that it does not increase the rate of monomer addition, decrease it by filament capping activity nor induce filament depolymerisation.

The recovery of the viscosity of actin filaments which have been fragmented into short segments and then allowed to anneal is greater in the presence of transgelin and leads to gelation. In the absence of transgelin, actin filament fragments do recover viscosity, by annealing, but do not gel. Whether transgelin is involved with this annealing of actin filaments or whether it crosslinks filaments once they have reformed into longer filaments is unclear. Transgelin-induced gelation of actin filament fragments was slower than with actin monomers, this may be due to the reduced mobility of fragments compared with monomers within the capillary tube as they aligned and annealed into filaments.

The role of filament annealing *in vivo* is not yet known. It may constitute a repair mechanism, since *in vitro* models have demonstrated that myosin can exert enough force to break an actin filament. It is possible therefore that filaments may "break" in muscle contraction and cell movements. Annealing of filaments that have been severed by proteins, such as gelsolin, may contribute to the rapid rearrangements of actin filaments that are associated with cell motility and shape changes. The finding that, *in vitro*, the joining of actin filaments can occur through direct association of the filaments ends, with no requirement for ATP hydrolysis, addition of actin monomers or accessory proteins does not preclude the presence of a protein that enhances annealing *in vivo* (311) Transgelin does not appear to behave as a barbed end capping protein such as radixin, which would

inhibit the recovery of viscosity of fragmented actin filaments (439), given the observed increase in viscosity of actin fragments in the presence of transgelin, but it may stabilize actin filaments by preventing their depolymerisation without affecting assembly as shown for a 30kDa protein from *D. discoides* (502) or cross link filaments that have spontaneously annealed.

Viscometric and light scattering assays, however, strongly suggest a bundling or cross linking role for transgelin in the formation of an actin gel. In low shear viscometry assays transgelin increased the viscosity to gel point, and gelation was not seen in actin alone. Transgelin, therefore, was not accelerating the completion of actin filament polymerization, but appeared to be increasing the viscosity of actin filaments to a point that they could reach in the absence of transgelin. This suggests that transgelin induces actin gelation either by crosslinking the actin filaments, or by stabilizing existing actin filament self associations (163).

Actin filament solutions form three dimensional networks of linear actin polymers and their viscosity is proportional to the polymer concentration, the length of the filaments and their orientation, which affect the degree of entanglement and self association of the polymers (416). Hence, control of cytoplasmic gelation (or gelation *in vitro*) can be achieved by any mechanism which alters the number of crosslinks or the number or length of actin filaments. Cytoplasmic gels such as actin crosslinked, for example, by ABP 280, can be described in terms which predict a sharp transition of actin from a viscous liquid to a solid gel at the critical gelling concentration (327), that is, the minimum concentration of crosslinker required to connect all the polymers into one continuous network (416). This sharp transition from a liquid to a gel as a function of crosslinker concentration is characteristic of many actin crosslinking proteins as they induce a gelling actin network (163, 416), and has been demonstrated as a feature of transgelin-induced actin gelation. The critical gelling concentration depends on a number of factors - the ratio of crosslinker to polymer, the gel point occurs at a critical ratio of crosslinker to monomers, and higher ratios propagating the network and increasing its rigidity; the length of the polymers; the functionality of the crosslinker, that is how many filaments each molecule can crosslink, so that as the functionality of a protein increases so does its potency as a crosslinker; the

affinity of the crosslinker for the filaments; and the distribution of the crosslinker (416). In practise a predicted value for the critical gelling concentration holds to a rough extent, but errors might be expected from uncertainty as to the actual concentration of functional crosslinks in the network, and because the theory assumes all bonds between polymers are formed by crosslinking proteins, whereas with actin, some crosslinks are formed by direct actin : actin bonds (416,352).

Increasing the concentration of transgelin above the concentration at which it induces actin gelation has no detectable effect in an falling ball viscometry assay, but may increase the rigidity or strength of the gelled network by forming further crosslinks. It may be possible to investigate this either by yield strength experiments where capillary tubes of gelled actin are centrifuged and the passage of the ball-bearing down the tube measured (352), or by electron microscopy to investigate whether there is an increased number of crosslinks.

A light scattering assay also showed that the actin was crosslinked into a more complex form in the presence of transgelin, with more light scattered in the presence of transgelin than in actin alone.

These data were extended and reinforced by using electron microscopy which showed that transgelin rapidly induced the formation of a gelled filament meshwork within the same time required for the rapid increase in viscosity seen in falling ball viscometry and light scattering assays.

A rise in pH occurs in quiescent cells when they are stimulated by growth factors or tumour promoters, and this alkalinization is necessary for the initiation of DNA synthesis. Cell stimulation also induces rapid alterations of actin cytoskeletal structures, and it has been suggested that a change in pH induces the reorganization of actin by modifying the activity of actin associated proteins, such as cofilin (500), and hisactophilin (167).

The gelation of actin by transgelin was unaltered by varying pH between 5 and 10. In contrast, cofilin reversibly controls actin polymerization and depolymerisation in a pH sensitive manner, so that at pH8.2 actin in the presence of cofilin completely depolymerizes, at pH 7 cofilin binds to F actin at 1:1 molar ratio, and induces partial depolymerization and at pH6.6 cofilin promotes rapid actin polymerization (500).

Neither the presence nor absence of Ca^{2+} prevented transgelin induced actin gelation.

13 Control of Actin /Transgelin Interaction By Ionic Conditions

The interactions of several actin associated proteins with actin are controlled by ionic strength. For example, ezrin has no actin binding activity at physiological salt concentrations (43). Fodrin binds and crosslinks actin, but by falling ball viscometry it was demonstrated that increasing the concentration of KCl above 100mM inhibited fodrin-induced actin gelation (143). Similarly fimbrin, which bind to and bundles actin in a salt dependent manner so that binding is optimal at 30mMKCl and is inhibited above 100mM. Fimbrin's interaction with actin can be stabilized at 50mM KCl with PEG (142, 42).

By falling ball viscometry it was found that the activity of transgelin was controlled by ionic strength, and that the ability of transgelin to induce actin gelation was totally abolished when 10 mM KCl was added to the polymerization buffer. This was not due to the rupturing effect of chaotropic Cl^- anions, since the elevation of ionic strength with potassium phosphate had the same effect, but to a general ionic (rather than a specific KCl) effect. on the cross links between actin and transgelin rather than on actin filaments themselves (163) Although some K phosphate buffer was used in the polymerization buffer, it was not used routinely to increase the ionic concentration above that of the control, as phosphate may lower the pointed end critical concentration and thus affects polymerization rates (432). Similar results have been found for the complex between 21kDa/23kDa NH_2 terminal fragments of myosin and actin, which is controlled by ionic strength in a manner similar to actin : transgelin (310).

Light scattering assays also reveal that transgelin induced actin gelation can be reversed by the presence of 100mM KCl. The increase in light scattering seen when actin is polymerised in the presence of transgelin is due to the formation of crosslinks/tangles which in turn leads to the formation of a gel which scatters more light than actin filaments alone. The decrease in light scattering at 100mM KCl indicates that transgelin-induced crosslinks or tangles are not formed, and that the transgelin/actin complex has dissociated.

Immunofluorescence experiments, where purified transgelin is added back to permeabilized cells from which all detectable transgelin had previously been extracted confirms the finding that at increased ionic strength, loss of transgelin-induced actin gelation is due to the absence of binding rather than transgelin remaining bound, but functionally inactive, in the presence of 100 mM KCl *in vitro*.

The ionic conditions which favour transgelin induced actin gelation are not those found in the cell and while I as yet have no explanation for this discrepancy, the cooperative nature of actin associated protein interactions (457,114), or the possibility that transgelin is part of a regulatory system that is highly sensitive to ionic conditions may explain this apparent discrepancy. It has been demonstrated that other actin associated proteins that bind to actin *in vivo* (338), and show gelling activity in low ionic conditions (163), are unable to gel or bundle actin *in vitro* in conditions which are thought to more closely resemble those found inside the cell (163, 42). For example, aldolase, a glycolytic enzyme, is associated with actin microfilament networks in cells, which may impose structural order on the glycolytic pathway. The interaction between actin and aldolase has been shown to be specific by histochemical, biochemical, ultrastructural and immunofluorescence techniques (338). *In vitro*, gelling activity demonstrated for aldolase is lost with a decrease in viscosity following the addition of 100mM KCl (163).

It is possible that interactions between components of the actin filament network may be modulated by local changes of ion concentrations in the cytosol, as the addition of macromolecules can induce local imbalances in osmolarity and osmotic stress by sequestration of water (84, 127,159) and it has been demonstrated that 20% ethandiol could stabilize the association between transgelin/C4¹ and actin thin filaments even in the presence of 50mM KCl, when they, and other actin associated proteins such as caldesmon and tropomyosin, dissociate at the same ionic concentrations in the absence of ethandiol (399). The interaction of fimbrin with actin filaments is stabilized by the presence of polyethylene glycol (42).

14 Actin Organization

Actin filaments are organized into many different and dynamic forms and can be cross linked or bundled in a variety of ways inside a cell (82). Single actin filaments serve as tracks for myosin based motility, highly cross linked polar filament bundles support the plasma membrane in surface protrusions such as microvilli and filopodia, more loosely packed antiparallel filaments in stress fibres interdigitate with myosin filaments and produce contactile forces and three-dimensional isotropic gels in the cortex define cell shape (100,170,417). The organization of actin in a cell is largely a function of its associated proteins and given the large repertoire of actin structures found, and the additional roles that many crosslinking proteins have, for example tethering the actin cytoskeleton to other cell structures, it is not suprising that they are vary in size and shape, each with a specialised role (100). Despite their often similar behaviour *in vitro*, the *in vivo* function of actin binding proteins is likely to be more diverse. This diversity of form also allows for differential regulation of actin binding proteins by for example, Ca^{2+} , phospholipids and protein cofactors (such as calmodulin) (100,455). The presence of several actin crosslinking/bundling proteins within the same cell suggests a degree of functional redundancy or that control of actin stability and organization is complex and requires the activity of a range of proteins (38).

Falling ball viscometry, light scattering and electron microscope studies demonstrate a crosslinking/aggregating role for transgelin in the formation of actin gels *in vitro*. Furthermore, electron microscopy showed that transgelin, did not induce the formation of actin filament bundles, which would have been indicated by the orientation of actin filaments into parallel arrays. In contrast, electron micrographs of fimbrin/actin clearly show that fimbrin crosslinks F actin into compact straight bundles (42), and that α actinin also crosslinks and aligns F actin into parallel bundles (212). Electron microscopy also allowed me to exclude the formation of an isotropic network of actin filaments by transgelin, such as that formed in the presence of ABP 280, a long flexible crosslinking molecule. Instead, dense tangles of actin, aggregated into tight foci and lacking any apparent pattern were found. This type of dense, tangled meshwork might be expected for

actin filaments cross linked by a small actin binding protein, and has also been demonstrated by a 36kDa actin binding protein from *D.discooides*. (328)

Although a number of crosslinking proteins are large molecules, there is at least one other protein of similar mass to transgelin which is known to function as an actin crosslinker, and induce gelation in actin filament solutions, *Acanthamoeba* gelactin I, a 23kDa monomer (289). The 30 kDa actin bundling protein from *D. discooides* is also a monomeric crosslinking protein (502).

While these data do not appear to correspond with transgelins distribution on linear actin stress fibres, the binding of transgelin to these actin bundles occur after their initial alignment into parallel arrays which are under tension due to attachment to the plasma membrane/substrate. Transgelin might then crosslink and reinforce the filament bundle structure (56,229). This suggestion can be investigated by microinjecting polyclonal anti-protein C4 antibody to sequester transgelin/C4¹, collapsing actin filaments by cytochalasin and looking for any delay in the reformation of stress fibres or alterations in transgelin's distribution. Inside the cells, other factors might constrain transgelin's activity as a 'tangler', such as the cooperative binding of other proteins (C4¹ for example), and organize transgelin/actin complexes into the more ordered structure of stress fibres. Transfection of cDNA encoding transgelin or microinjection of the protein into cells where stress fibres are absent might also demonstrate a potential role for transgelin in stress fibre organization or formation. Although I have defined that the *in vitro* role of transgelin is to induce actin gelation, and this cannot be ruled out as its *in vivo* role, it is equally possible that in the cell transgelin has different and/or additional roles. For example, ABP 50 which binds to actin has also been identified as EF 1 α (495)

15 Mechanism of Transgelin Induced Gelation

A major possibility arising from these studies was that the gelling activity of transgelin was controlled via the formation of dimers or oligomers at low ionic strength. To investigate this I used linear sucrose gradients, in which the sample to be studied is layered onto the gradient, and materials with different sedimentation properties separate from each other during centrifugation (288). To prevent potential non-specific interactions between

transgelin and protein standards which would be a source of error, in this study the protein standards and transgelin were all loaded in separate gradients.

The approximate native molecular mass of a protein can be determined by a simple ratio of mobilities when well characterized proteins are included as standards. The viscosity and density of this sucrose gradient at 4°C gives an essentially linear migration of most biological materials, therefore the ratio of distances travelled from the meniscus by any two substances will always be constant (288). The Mr of transgelin remains 21 kDa irrespective of ionic conditions, banding in the same position as soy bean trypsin inhibitor, a 21 kDa monomeric molecule. Transgelin, therefore, does not form a dimer in the ionic conditions in which it induces actin gelation. Although the full length cDNA clones coding for transgelin reveal that transgelin has one free cysteine residue which would allow dimerization to occur (357), the folding of the protein may make this residue inaccessible or it may be blocked by glutathione in the cell (5). The NH₂ terminus of caldesmon carries an exposed SH group which forms a disulphide bridge with the SH group of a second caldesmon molecule, resulting in a dimer that is capable of crosslinking and bundling actin filaments (284, 46). A highly reactive cysteine residue might be expected to interact with free cysteine residues in other molecules such as actin, which has five cysteine residues (79). However transgelin is detergent soluble in non reducing conditions, and can be competed off by polyanions, showing that the interaction is likely to be electrostatic. It is possible that binding to actin induces oligomerization of transgelin, possibly by altering the conformation of transgelin to allow it to form self dimers, (the cooperative binding of insertin (372) and hisactophilin (236,167) molecules via an actin filament has been documented) and I cannot yet exclude this possibility. Local high concentrations may induce dimerization of transgelin, as has been demonstrated for talin and vinculin (269,445) Other modifications, such as phosphorylation or the presence of other actin associated proteins may also allow dimerization. It is possible that transgelin may form a dimer with C4^l, and this will be investigated by running a linear sucrose gradient (288) containing both purified proteins or by equilibrium column chromatography (189). Since transgelin remains as a monomer in a variety of ionic conditions and actin crosslinking requires the presence of two binding sites, with each binding to a separate actin filament it

is possible that transgelin itself has two actin binding sites (292). For example, ABP 50, which crosslinks actin filaments into bundles exists as globular monomer and has been two actin binding sites (292).

Another possibility for the mechanism of transgelin-induced actin gelation is that transgelin might act to protect or stabilize the weak cytochalasin sensitive interconnections that are thought to occur between actin filaments as they cross over and come into close contact with each other (163,352). Some mechanism to control these self associations might be required by the cell, as they can be sufficiently strong for the viscosity of pure column-purified actin filaments in solution to reach infinity at the low shear rates found inside cells (163,416, 275). It is also possible that transgelin might bind to actin and modify the neutralise the charged groups that are present in the filaments, enabling the filaments to come into close contact with each other. The ordered helical structure of actin filaments minimizes the intramolecular neutralization of positive and negative charges and promotes the juxtaposition of these opposite charges between neighbouring filaments; transgelin enhance this effect (416). Finally, transgelin may, like calponin (322), induce a conformational change in actin molecules within a filament, and enhances filament self-association. Transgelin may have one actin binding site, while other regions affect gelation by masking charged residues.

16 Molecular Mechanism of Transgelin/Actin Interaction

Analysis of the amino acid sequence and of the full length cDNA clones coding for transgelin reveal that the transgelin molecule contains an interspersed cluster of 5 positively charged amino acids, 3 lysines, 1 arginine and 1 histidine in an 8 residue sequence between residues 156-161, (KKAQEHR) (357). This is the only positively charged cluster of amino acid residues and it is possible that it represents an actin binding site, that can be blocked by increasing ionic strength. To further investigate the nature of transgelins interactions with actin, I blocked this site with the polyanion, sodium tripolyphosphate. Falling ball viscometry clearly showed that the addition of 1mM sodium tripolyphosphate not only totally abrogated actin gelation induced by transgelin, but reduced the viscosity of actin + transgelin to control levels. This sensitivity to ionic strength, leading to transgelins

dissociation from actin, suggests that either the actin binding site or the functional gelation site (another actin binding site, or a self association site) of transgelin is associated with these positive residues. The 21kDa and 23kDa N terminal fragments of myosin similarly bind to actin in an ionic strength dependent manner, and they contain a single cluster of 4 positively charged residues in the 143-147 stretch with the sequence Arg-Gly-Lys-Lys-Arg. This region may participate in the actin binding of myosin since specific antibodies against this site inhibited the interaction of native S1 with actin, and it is the only positive charged cluster in the amino acid sequence of myosin (310). The interactions between myosin and actin, and transgelin and actin are likely to be electrostatic in nature, with the effect of KCl and sodium tripolyphosphate based on competition of these anions/polyanions with actin for the cluster of positively charged amino acids (310,455). Non coulombic forces are probably less involved. KCl and the polyanionic polyphosphate show a direct correlation between the number of net charges to their efficiency of dissociation and their effect is probably due, not to a specific structure, but the density of negative charges (310). Specific antibodies against the positive amino acid cluster in transgelin, and the use of synthetic peptides corresponding to this region in *in vitro* competition assays will be used to determine if this area of positively charged amino acid residues is an actin binding site. Structural elements may contribute noncoulombic forces to actin binding of transgelin, as occurs in myosin S1 (310).

Using a zero length carbodiimide cross linker with a variety of actin binding proteins, it has been shown that the region of the actin molecule to which these proteins preferentially crosslinked is the very acidic NH₂ terminus, residues 1-12, consisting of 4 (muscle actin) or 3 (non muscle actin) acidic residues. Some of the molecules that have been crosslinked to this region of actin - myosin head, depactin, fragmin, cofilin, α actinin, troponin I, gelsolin terminal NH₂ domains and actolinkin, have been sequenced and these show no homology between each other except that they are all very rich in basic amino acid residues. This suggests that the actin NH₂ terminus may function as a dominant negatively charged area attracting a positively charged cluster at the complementary surface of the actin binding protein (455). LSP-1 binds to F actin via its basic CO₂H terminal domain. A

number of other basic proteins have been shown to bind to actin, for example aldolase and *acanthamoeba* gelactins (163).

The molecular nature of the putative second actin binding site of transgelin is at present undefined. While several distinct protein structures have been identified as actin binding domains, for example, that found in the head domains of myosins, and the α actinin family, the sequence of many other actin binding proteins do not show homology with each other or with identified actin binding domains (236,459).

Sequence data has shown the presence of a motif (LKAAED) in transgelin that is similar in sequence to an actin binding site found in actobindin, thymosin β 4, α actinin, fimbrin and tropomyosin and may represent an actin binding site in transgelin (357,459,453). This possibility will be investigated, initially with peptides in competition assays.

17 Stoichiometry of Transgelin /Actin Binding

To investigate the stoichiometry of the actin transgelin interactions discussed above I used co-sedimentation assays (492). Apparent saturation was achieved at an approximate ratio of 1 transgelin to 6 actin monomers, which is in agreement with data showing that 1.5 μ g protein C4 was isolated from 1×10^6 fibroblasts, which are thought to contain 6 μ g actin, (174), and is of the same order as caldesmon or nonmuscle tropomyosin : actin (1: 6) (133,72,465). The apparent binding constant of $7.5 \times 10^5 \text{M}^{-1}$. This binding constant is similar to non muscle tropomyosin, which is $4.4 \times 10^5 - 1.5 \times 10^6 \text{M}^{-1}$ (492). Transgelin : actin binding is saturable which indicates that it is specific. The concentration of transgelin (2.4 μ M) required to gel 9.3 μ M actin is slightly more than the apparent saturation concentration found in these binding studies. Even so, ratio of transgelin : actin (1 : 4) at which transgelin induces actin gelation is less than that of an actin binding protein found in *Limulus* which binds 1:1 with actin (432a) and similar to dematin where at saturation 1 trimer associates with 10 - 12 actin monomers, or 3 dematin monomers:10-12 actin (192). Fimbrin binds 1:2 or 3 monomers and high density is important for the rigidity of the tightly packed bundles that it forms in microvilli (142). Calponin also binds with a maximum stoichiometry of 1:3 (465)

The binding constant found for transgelin *in vitro* and its detergent solubility does not necessarily indicate a minor *in vivo* role for transgelin, for example in stress fibre function or organization, since relatively low affinity binding has been demonstrated between integrin and talin, proteins in the complex which anchors microfilaments to the plasma membrane and form links between the extracellular matrix and the cytoskeleton (189). Nor do apparently weak interactions *in vitro* necessarily exclude the possibility of a stronger association *in vivo*, in the presence of all components in a putative molecular cascade of which transgelin is only a part. For example, multiple low affinity interactions occur between the numerous components of the adhesion plaque and maintain the attachment between integrins and stress fibres (443). Low affinity actin binding may enable actin : actin binding proteins bonds to be broken and reformed very rapidly and allow the dynamic actin cytoskeleton to remodel and change shape at a rate compatible with observed cell activity. The formation of actin tangles by transgelin suggests that it cross links actin by multiple weak bonds that may have an additive effect to form a strong complex. High concentration, high valency, low affinity binding may have advantages in the cell, forming a complex that is easily formed or broken, and might provide for slippage in the contraction of stress fibres. Gels of actin filaments and α actinin are much more rigid when deformed rapidly than slowly, presumably because the cross links can rearrange if given sufficient time (378,236).

The binding constants of some proteins *in vivo* may be greater than the constants in a dilute solution due to the effects of high protein or other macromolecules concentrations on the physical states of water inside the cell and the resultant osmotic imbalances (89,127,84). In high protein concentrations, water can exist in two forms, one freely diffusible and the other, water of hydration, a semi liquid which forms a shell around proteins and prevents their free diffusion, thus enhancing protein:protein associations (89,127). Alternatively phosphorylation of transgelin may change its affinity for actin, or the binding of another protein (in this case possibly C4^l) may modify transgelin's interactions with actin and alter its affinity or activity in a manner analogous to caldesmon which enhances the binding constant of tropomyosin to actin from 4.4×10^5 to 1.5×10^6 M⁻¹ (492)

The interaction of transgelin with skeletal muscle actin is not surprising even although it is clearly absent from this tissue, given the high degree of conservation between actin isoforms in different tissues and widely divergent species (230,414,476,348). It would be interesting to determine the binding constant and saturation concentration of transgelin with actin derived from smooth muscle or non muscle cells. Studies with other actin associated proteins for example thymosin b4 and profilin bind non-muscle actin in preference to muscle actin (180) and high Mr tropomyosins demonstrate a higher affinity for α muscle than non muscle β and γ actins (262).

18 Regulation of Transgelin by Calcium

Increasing the intracellular level of Ca^{2+} in response to external stimuli triggers many cellular responses, including reorganization of the actin cytoskeleton (90,179,392). Many actin associated proteins that control actin polymerization and organization are regulated by Ca^{2+} , either by directly binding Ca^{2+} , for example α actinin, or via the activity of another Ca^{2+} binding protein, for example calmodulin and caldesmon (455)

To investigate whether or not transgelin binds Ca^{2+} I incubated the purified protein with $^{45}\text{Ca}^{2+}$ after SDS PAGE/immunoblotting (291). This revealed the presence of a known calcium binding protein (parvalbumin) as a radioactive band. This method allows the detection of as little as 2 μg of known calcium binding proteins, including those which bind calcium only weakly. This technique has shown Ca^{2+} binding by troponin C, calmodulin, fibulin and parvalbumin and demonstrates that calcium binding proteins will retain calcium binding activity even after SDS PAGE electrophoresis and electrophoretic transfer (291,9). In the present study it was shown that while parvalbumin binds Ca^{2+} strongly, transgelin does not bind Ca^{2+} even weakly. This is in agreement with the finding that (a) transgelin induced actin gelation is independent of Ca^{2+} and (b) our cDNA studies which show that transgelin has a redundant EF hand, (357). It would be interesting to look for Ca^{2+} binding in yeast where transgelin is present in abundance to see if the ability to bind this important regulatory cation has been lost in evolution.

While transgelin's interactions with actin *in vitro* are calcium insensitive and it cannot itself bind calcium, calcium may regulate transgelin activity in a complex/cascade of

proteins via the action of another calcium sensitive protein.. For example, caldesmon, binds to F actin in a calcium independent manner, and calcium regulation is introduced by calmodulin, so that in the presence of calcium, calmodulin binds to caldesmon and inhibits its binding to F actin, whereas in the absence of calcium, calmodulin does not bind caldesmon which is free to bind to actin and inhibit actomyosin ATPase (455,465).

19 Transgelin Rebinding Assay

Since sedimentation assay data shows that transgelins binding is saturable and therefore specific, and falling ball viscometry, light scattering and electron microscopy defined the molecule functionally as an actin gelation factor, suggesting that the purification protocol did not denature the protein, I decided to develop an *in vitro* immunofluorescence rebinding assay which was rapid and sensitive, since as few as 5000 molecules can be detected on the surface of a lymphocyte (95a). In addition to visually investigating the actin binding activity of transgelin and further assaying its functional integrity, this assay gives specific positional information about the rebinding of transgelin to actin. Furthermore, this assay helps to determine whether or not the loss of transgelin-induced actin gelation in elevated KCl is due to loss of transgelin : actin binding or functional inactivation of one or more actin binding sites. For example, it was possible that one of the two putative actin binding sites necessary to cause gelation was salt sensitive while the other was not. This has been found for a caldesmon-like 210 kDa protein present in *Physarum* which has two actin binding sites, only one of which is sensitive to ionic conditions, but both are required for actin bundling activity (205). Similarly, in transgelin both actin binding sites would be required for actin gelation, but only one for binding.

Coverslips of REFs, with large stress fibre bundles from which all detectable endogenous transgelin had been removed, were incubated in different ionic conditions with purified transgelin (at different concentrations) and examined by immunofluorescence. These data reinforced my biochemical and functional studies and showed that transgelin rebound specifically to F actin filaments in a distribution and at levels indistinguishable from control cells. The rebinding of transgelin in these experiments was dependent on ionic conditions, being lost at salt concentrations greater than 12 mM, which is fully in

agreement with the results of falling ball viscometry and light scattering assays. Furthermore, saturation of transgelin binding at $3\mu\text{M}$ is in agreement with sedimentation assays.

This rebinding assay also demonstrates that (a) it is lack of binding, and not functional inactivation of a single salt sensitive crosslinking site, that accounts for the loss of transgelin induced actin gelation at elevated ionic conditions, and (b) both sites must therefore be sensitive to ionic conditions since transgelin can neither bind to actin, nor gel actin at high salt.

FUTURE WORK

Transgelin binds to and induces actin filament gelation, but at present actin binding sites on this molecule have not been identified. Two potential sites for transgelin : actin interaction are the cluster of basic amino acids which may interact with actin in an ionic strength dependent manner, and an amino acid sequence LKAAEDY, which shares homology with regions in actobindin and thymosin β 4 which have been shown to interact with actin. Similar motifs have been found in α actinin, tropomyosin, fimbrin and myosin heavy chain. Synthetic peptides corresponding to these regions of transgelin, or antibodies raised against them, could be used in a variety of functional assays, such as falling ball viscometry, light scattering, and rebinding in a permeabilised cell system to see if they compete with transgelin for binding sites on actin, and therefore inhibit transgelin activity.

Interactions between transgelin and other actin associated proteins, such as C4¹ and tropomyosin can be investigated using either linear sucrose gradients or equilibrium column chromatography while regulation of transgelin's activity as an actin gelation factor can be studied by testing the effect of transgelin in the presence of other actin associated proteins in functional assays, such as falling ball viscometry, light scattering, and rebinding in permeabilised cell systems.

I would also like to purify C4¹ and study its *in vivo* and *in vitro* functions, initially using the assays used in this thesis.

The *in vivo* function of transgelin has not yet been defined. This can be investigated by transfecting the gene encoding transgelin into cells where it is absent, such as transformed fibroblasts and looking for changes in the transformed phenotype, such as altered morphology, increased anchorage dependence, contact inhibition, the formation of actin stress fibre bundles and an inability to grow in soft agar.

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