Studies on the role of annexin II in rat basophils

~~Annexin II and exocytosis~~

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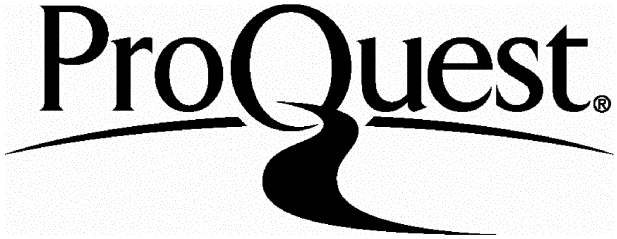

Abstract

The annexins are a family of calcium-dependent phospholipid-binding proteins. They are defined by the presence of a highly conserved 70 amino acid domain responsible for binding calcium ions and interacting with phospholipid membranes. All annexins contain four copies of this repeat except for annexin VI, which has eight. In addition, each annexin has a unique N-terminus, varying in size from around 10 to nearly 200 amino acids. Ten different annexins have been cloned in mammals and a further three in lower eukaryotic phyla. No functions have been unequivocally assigned to any members of the family. Most annexins have a restricted tissue distribution, but all cells express at least one member of the family.

A secretory cell line, rat basophilic leukaemia RBL-2H3 cells was shown to express annexins I, II, VI and VII, all of which have been implicated in exocytosis. Immunolabelling of annexin II in RBL-2H3 cells, revealed that it is localised to the cortical region and to punctate bodies in the cytosol. The presence of annexin II close to the plasma membrane, and possibly on secretory vesicles located in the cytosol, is consistent with a role in exocytosis.

Several rat annexin II cDNA clones were isolated using a murine annexin II cDNA to screen a plasmid cDNA library. One of these was fully sequenced. Alignment of the rat annexin II cDNA sequence with those from other species revealed a 6-nucleotide insert in the coding region of one of the clones, close to the N-terminus. The insertion of 2 amino acids at this point may have important functional consequences since it is close to the sites of phosphorylation and the region that interacts with annexin II's cellular ligand, p11. To demonstrate the existence of this insert *in vivo*, primer extension analysis was performed on mRNA extracted from RBL-2H3 cells.

To investigate the effect on secretion of a reduction in annexin II expression, RBL-2H3 cells were transfected with an expression plasmid containing rat annexin II cDNA in reverse orientation. A clone expressing significantly lower than wild type levels of annexin II was shown to have reduced secretory response to Ca^{2+} and GTPγS. Over a period of weeks this clone reverted to wild type levels of annexin II expression, and this coincided with a return to normal secretory response. The results in this thesis support the theory that annexin II functions in the secretory pathway.

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List of Abbreviations

AA	arachidonic acid
ADP	adenosine 5' diphosphate
ARF	ADP ribosylation factor
ATP	adenosine 5' triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine 5' monophosphate
DAG	diacylglycerol
DEAE-dextran	diethylamino-ethyl-dextran
DMEM	Dulbecco's modified essential medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
EMEM	Eagle's modified essential medium
F-actin	filamentous actin
FCS	foetal calf serum
GDP	guanosine 5' diphosphate
GTP	guanosine 5' triphosphate
GTPγS	guanosine 5'-(3-*O*-thio) triphosphate
HBS	HEPES-buffered saline
HEPES	N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid
hexosaminidase	N-acetyl-β-D-glucosaminidase
IgE	immunoglobulin E
IgG	immunoglobulin G

IP_3	inositol 1,4,5 trisphosphate
ISA	intestinal-specific annexin
LB	Luria broth
LTR	long terminal repeat
M_r	relative molecular weight
mRNA	messenger ribonucleic acid
MVB	multivesicular bodies
Neo	neomycin
NGF	nerve growth factor
PBS	phosphate buffered saline
PA	phosphatidic acid
PC	phosphatidylcholine
pCa	$-\log_{10}[Ca^{2+}]$
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PIP2	phosphatidylinositol-4,5 bisphosphate
PIPES	piperazine-N,N'-bis(2-ethane-sulphonic acid)
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulphonylchloride
PS	phosphatidylserine
RSV	Rous sarcoma virus
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SL-O	streptolysin O
TPA	12-*O*-tetradecanoyl-phorbol-13-acetate
Tris	Tris(hydroxymethyl)methylamine

Chapter 1

Introduction

1.1 Calcium and the cell

A link between calcium and cell function was first recognised by Sydney Ringer in 1883, who found that to maintain contractions in an isolated heart it was essential to have calcium salts in the bathing solution. About 60 years later scientists on different sides of the globe observed that injection of calcium ions into muscle fibres caused them to shorten (Kamada and Kinosita, 1943; Heilbrunn and Wiercinski, 1947). The mechanism of muscle contraction is now well understood and results from a conformational change in troponin C, induced by the binding of calcium ions. A rise in intracellular calcium ion concentration has been shown to govern many other cellular processes including exocytosis, cell division, motility, ion transport and many enzyme activities depending on cell type. Fine control of intracellular calcium levels is extremely important for some processes such as neurite outgrowth, where too much or too little calcium is inhibitory (Al-Mohanna *et al.*, 1992), and loss of control of calcium regulation can result in neurodegenerative cell death (Tymianski *et al.*, 1993).

The central role of calcium in the regulation of many cellular processes is mediated by its interaction with a variety of proteins (Carafoli and Penniston, 1985). Many calcium receptor proteins have been identified and we have begun to understand how calcium binds to these proteins and alters their properties. As a consequence of this, calcium-binding proteins have received much attention.

1.2 Calcium-binding proteins

The best known calcium-binding motif is the "E-F hand" which consists of a continuous stretch of 12 amino acid residues forming a helix-loop-helix structure (Tufty and Kretsinger, 1975). Probably the best known example of this motif is in the ubiquitous protein calmodulin, which contains four E-F hand type calcium-binding

sites. Recently an alternative more complex calcium-binding site has been identified and characterised as unique to members of the annexin family (the calcium-binding site of phospholipase A_2 has a similar conformation although it shares no sequence homology with the annexins (Fleer *et al.*, 1980)). The positive identification of this structure was made possible by the solution of the tertiary structure of annexin V (Huber *et al.*, 1990a) which is described in detail below. Previously it had been assumed that a particularly highly conserved stretch of 17 amino acids in the annexin family, known as the "endonexin fold", comprised the entire calcium binding site. This however has been shown to be an oversimplification. In fact the Ca^{2+}-binding site is formed by several non-consecutive residues (and phospholipid head groups when bound to membranes). The properly folded structure of an entire 70 amino acid calcium-binding domain found in the annexin family is necessary to provide all the calcium co-ordinating ligands (see section 1.3.1).

This introduction will review properties and possible functions of the whole annexin family, before concentrating on annexin II. The second part (from section 1.7 onwards) covers aspects of the process of secretion, in which annexin II has been suggested to function, mainly focusing on the basophilic cell line used for the work in this thesis.

1.3 The annexin family

The annexins made their first appearance in the scientific literature in 1978. Annexin VII, then called synexin, was identified as a protein that could aggregate adrenal medullar chromaffin cell secretory vesicles *in vitro* (Creutz et al., 1978), and it was proposed that its function was to fuse vesicles to the plasma membrane or to each other (in compound exocytosis). Since then another nine mammalian annexins, and several more in lower eukaryotes, have been discovered and many other possible functions have been proposed (Crompton et al., 1988).

The defining characteristics of the annexin family are calcium-dependent phospholipid binding, and the presence of a conserved 70 amino acid domain that is responsible for calcium- and phospholipid-binding. All annexins contain four copies of

this conserved repeat (except for annexin VI which has eight) and a variable N-terminus which ranges in size from only 10 amino acid residues in annexin IV to 194 residues in annexin XI. The repeats are 40-60% identical to one another within each annexin, and family members share 45-55% amino acid sequence similarity with each other in the C-terminal core domain. The sequences of all known human annexins are aligned in figure 1.1.

The variable N-terminus of each annexin is thought to confer unique properties in addition to the characteristic shared properties contributed by the core domains. Many different functions have been proposed for the annexins, reflecting the fact that they have been independently isolated by workers from diverse fields (see section 1.4). They are expressed in a wide range of tissues and cell types, from spermatozoa (Berruti, 1988) to mammary epithelial cells (Schwartz-Albiez *et al.*, 1993) to muscle (Hazarika *et al.*, 1991). All cell types appear to express two or more members of the annexin family (Crompton *et al.*, 1988).

Annexin I was first isolated as a 35kD substrate of the epidermal growth factor-receptor (EGF-R) kinase in A431 cells (Fava and Cohen, 1984), and was later found to be identical to lipocortin, a phospholipase A_2 inhibitor from rat peritoneal exudate (Pepinsky and Sinclair, 1986; Huang *et al.*, 1986). Annexin II was first isolated as the major cellular target of $pp60^{v\text{-}src}$ tyrosine kinase, the transforming gene product of the Rous sarcoma virus (RSV) (Erikson and Erikson, 1980; Radke *et al.*, 1980). It was subsequently discovered to be identical to calpactin, a spectrin- and F-actin-binding protein from brush borders (Gerke and Weber, 1984), and also a phospholipase A_2 inhibitor protein (Huang *et al.*, 1986).

It was not until annexins I and II were cloned in 1986 that the internally repeated structure characteristic of the annexins became apparent. This was rapidly followed by the identification of annexin IV by peptide sequencing (Weber *et al.*, 1987) and molecular cloning (Hamman *et al.*, 1988; Grundmann *et al.*, 1988).

Annexin V was isolated as an inhibitor of blood coagulation (Reutelingsperger *et al.*, 1985), a property of all annexins that can be attributed to calcium-dependent sequestration of the phospholipid matrix essential for normal coagulation. Annexin VI,

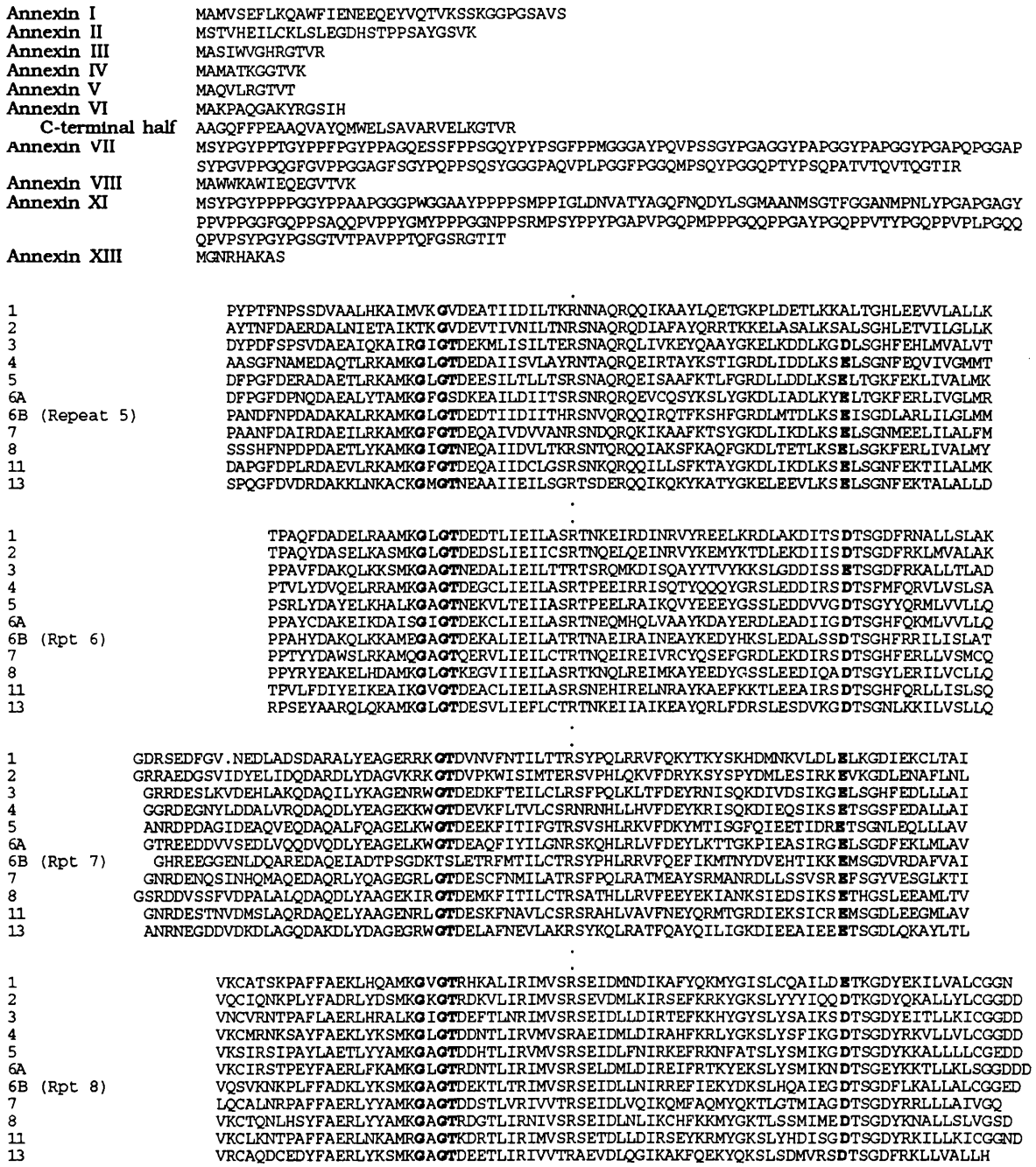


Figure 1.1 *Protein sequence alignment of all known human annexins (I-VIII, XI and XIII)*
The protein sequences are arranged reflect the repetitive domain structure of the annexins. The unique N-termini are followed by the four repeats of each annexin. The two halves of annexin VI, which has eight repeats, are included. The repeated domains are aligned with each other according to the position of an invariant arginine residue (R) indicated by the dots between repeats. Residues that conform to the calcium binding site consensus determined by Huber *et al.* (1990b) are shown in bold.

the only member of the family containing eight copies of the repeated domain, was first isolated and purified from the detergent-insoluble fraction of lymphocyte plasma membranes (Owens and Crumpton, 1984). Molecular cloning revealed the structural relationship between annexin VI and the other members of the family (Moss *et al.*, 1988). This eight-repeat annexin is likely to have evolved from a four-repeat annexin by a gene duplication event as repeat 5 is most similar to repeat 1, repeat 6 to repeat 2 and so on. Annexin III was also cloned in 1988 from rat and human (Pepinsky *et al.*, 1988).

Annexin VIII cDNA was detected by cross-hybridisation with oligonucleotides used to screen a human placental cDNA library for annexin V (Hauptmann *et al.*, 1989). However the tissue distribution of this annexin appears to be extremely limited and the protein product was not detected until 1991 in human placenta (Pepinsky and Hauptmann, 1991). An 88% identical protein has recently been isolated from rabbit lung and this is presumed to be the rabbit annexin VIII homologue (Tsao *et al.*, 1991). Annexin VII, although the first to be discovered was not cloned until relatively recently (Magendzo *et al.*, 1991).

Annexin XI was isolated from rabbit lung as a calcyclin-associated protein of 50kD and called CAP-50 (Towle and Treadwell, 1992; Tokumitsu *et al.*, 1992). The most recent to be discovered is an intestinal-specific human annexin, ISA, which has been cloned from a human colon adenocarcinoma cell line (Wice and Gordon, 1992).

Members of the annexin family have also been found in amphibians (Izant and Bryson, 1991) and fish (Walker, 1982), as well as higher plants (Smallwood *et al.*, 1990) and other lower eukaryotic phyla. Some are mammalian homologues: the annexin VII homologue from *Dictyostelium discoideum* (Doring *et al.*, 1991), and the annexin I homologue isolated from the sponge *Geodia cydonium* (Robitzki *et al.*, 1990). Others are unique: antibodies to a consensus peptide from the conserved endonexin fold region identified two proteins of apparent M_r 30 and 34kD in *Drosophila melanogaster* (Gerke, 1989a), these have since been cloned and assigned annexins IX and X (Johnston *et al.*, 1990); at least two annexins are present in *Hydra*, one has been cloned and named annexin XII (Schlaepfer *et al.*, 1992a; Schlaepfer *et al.*, 1992b). The list of annexin family members will almost certainly continue to grow.

The many different contexts in which annexins have been isolated, and the many possible functions tentatively attributed to them, spawned a diversity of names for the family members. In 1990 in an attempt to simplify the nomenclature, the generic term "annexin", originally coined by Geisow *et al.* (1987), was adopted by most investigators (Crumpton and Dedman, 1990) (see table 1).

1.3.1 Three dimensional structure of annexin V

The crystal structure of annexin V has been solved (Huber *et al.*, 1990a), and the calcium binding sites identified (Huber *et al.*, 1990b). Each of the four conserved repeats forms five α-helices (*a* to *e*) which are wound into a right-handed super-helix. These form a planar array around a central cavity, with repeat 1 lying next to repeat 4 to close the circle. Viewed from side on, the molecule has a slightly convex surface, from which protrude the Ca^{2+} co-ordinating ligands, and a slightly concave surface where the N-terminus lies. The structural conformation of the N-terminus has not been well defined, suggesting that it can exist in several positions, in keeping with the notion that it may have a regulatory role.

Huber *et al.* (1990b) report that there are three high affinity Ca^{2+}-binding sites located in repeats 1, 2 and 4 of human annexin V. Five of the co-ordinating ligands for each calcium ion are provided by carbonyl oxygens in the interhelical loop between helices *a* and *b* (these lie in the highly conserved "endonexin fold" region), and the carboxyl oxygens of an acidic amino acid located 39 residues C-terminal to these. The consensus sequence is Gly, X, Gly, Thr, 38 residues, Glu/Asp (residues conforming to this are indicated in figure 1.1). Solvent molecules provide a sixth ligand for the co-ordination polyhedron. An unoccupied seventh site is proposed to be filled by the phosphoryl moiety of an acidic phospholipid when the convex side of the molecule interacts with the membrane. A further two, low affinity Ca^{2+}-binding sites were located by soaking the crystals in solution containing lanthanum ions. They bear no resemblance to the high affinity sites and both reside in the first repeat where co-ordinating ligands are provided by glutamic acid residues. The third repeat has lost the first glycine of the consensus sequence and was not observed to bind Ca^{2+} in human

Table 1 *The multitude of names given to annexins isolated by different groups and the unifying nomenclature proposed by Crumpton and Dedman (1990)*

Annexin I	**Annexin II**	**Annexin III**	**Annexin IV**
Lipocortin I	Calpactin I	Lipocortin III	Endonexin I
p35	Lipocortin II	PAP-III	Protein II
Calpactin II	p36	35-α Calcimedin	32.5K Calelectrin
Chromobindin 9	Chromobindin 8		Lipocortin IV
GIF	Protein I		Chromobindin 4
	Placental anticoagulant protein-IV (PAP-IV)		PAP-II
			Placental protein 4-X (PP4-X)
			35-β Calcimedin

Annexin V	**Annexin VI**	**Annexin VII**	**Annexin VIII**
PAP-I	p68, p70, 73K	Synexin	VAC-β
IBC	67K Calelectrin		
Lipocortin V	Lipocortin VI		
35K Calelectrin	Protein III		
Endonexin II	Chromobindin 20		
PP4	67K Calcimedin		
Vascular anticoagulant-α (VAC-α)	Calphobindin II		
35-γ Calcimedin			
Calphobindin I			
Anchorin CII			

Annexin IX	**Annexin X**	**Annexin XI**	**Annexin XII**
(from *Drosophila*)	(from *Drosophila*)	CAP-50	(from *Hydra*)

Annexin XIII
ISA

annexin V (see figure 1.1), however Concha *et al.* (1993) reported two Ca^{2+} binding sites in repeat three of the rat annexin V crystal structure. The coordinating ligands for these sites are not conserved in human annexin V and may not be functional *in vivo*.

The funnel-shaped channel through the centre of the molecule is formed by four α-helices with its wider end at the convex surface. A small (4°) conformational change occurs when the calcium-binding sites are fully occupied. This is proposed to open the central channel, allowing Ca^{2+} to pass through (Concha *et al.*, 1993). Two possible salt bridges could be formed across the channel.

Annexin V appears to bind to membranes with little structural change. Mosser *et al.* (1991) report that annexin V binds to planar phospholipid monolayers at the air-water interface as a 2-dimensional array of repeated trimers, and suggest that the trimer may be the functional form of annexin V. Bound to large phospholipid vesicles, annexin V deforms the membrane into planar facets (Andree *et al.*, 1992).

The high degree of sequence conservation between members of the annexin family make it likely that the general structure of annexin V is common to all other annexins. This view is supported by secondary structure predictions indicating α-helices in the repeated domains of other annexins (Barton *et al.*, 1991).

1.3.2 Membrane binding and membrane aggregation by annexins

All annexins are capable of binding to phospholipid membranes in the presence of calcium. A study of annexins I to VI found that all have the same preference for phospholipid-binding: phosphatidic acid (PA) > phosphatidylserine (PS) > phosphatidyl-inositol (PI) > phosphatidylethanolamine (PE), and none binds phosphatidylcholine (PC) (Blackwood and Ernst, 1990). The extent of binding at different calcium concentrations varies between annexins, but all require Ca^{2+} in the micromolar range. In addition to simple binding, annexins I, II and VII can promote calcium-dependent aggregation of phospholipid vesicles and isolated secretory vesicles (Creutz and Sterner, 1983; Blackwood and Ernst, 1990).

The first annexin discovered to induce membrane aggregation was annexin VII. It *binds* to chromaffin granules at 5-10μM Ca^{2+}, but requires much higher Ca^{2+} levels

(200μM) to produce half-maximal aggregation (Creutz and Sterner, 1983). If we assume that annexin VII has the same number of Ca^{2+}-binding sites as annexin V, i.e. three high affinity binding sites and two low affinity, these results could be rationalised by hypothesising that occupation of at least one of the high affinity Ca^{2+}-binding sites enables the protein to bind to the membrane but further ions must be bound (possibly by the low affinity sites) before the annexin can induce membrane contact.

Clues to the mechanism of membrane aggregation come from studies showing that self-association of annexin VII occurred with the same calcium-dependency as membrane aggregation, (Creutz *et al.*, 1979). It was hypothesised that annexin VII on one membrane interacted with annexin VII on another. Indeed, for the planar membrane/calcium-binding face of a single annexin molecule to bind to two vesicles simultaneously would require a substantial distortion of either the protein or the two membranes concerned. In support of the mechanistic scenario of annexin molecules on adjacent membranes interacting to cause aggregation, phosphorylation of annexin I in the N-terminus (away from the membrane-binding face) by protein kinase C produces a slight decrease in the Ca^{2+}-requirement for phospholipid binding but a large increase in the Ca^{2+}-requirement for membrane aggregation (Schlaepfer and Haigler, 1987; Ando *et al.*, 1989; Wang and Creutz, 1992). In this respect it is interesting to note that annexin I isolated from rat cerebral cortex exists as two molecular forms, a monomer and a covalently-linked dimer associated with the synaptic plasma membrane (Pradel and Rendon, 1993); placental human annexin I also has a covalently-linked dimeric form (Pepinsky *et al.*, 1989). This indicates that at least in these tissues the dimerised form has an important role, though this may be different from that of the monomer.

Although it is established that the repeated domains of annexins are involved in binding to phospholipid membranes it is not clear why only a subset of the family can mediate vesicle aggregation. Ernst *et al.* (1991) used a monoclonal antibody to repeat 1 of annexin I which inhibits aggregation but does not prevent phospholipid binding, to identify the first repeat of annexin I as essential to its ability to promote vesicle aggregation. In addition, a chimera consisting of the first repeat of annexin I (residues 41-118) fused to repeats 2, 3 and 4 of annexin V exhibited vesicle aggregating activity,

whereas the wild type annexin V was inactive (Ernst *et al.*, 1991). It is not clear why repeat 1 of annexin I should confer this activity but perhaps it mediates self-association. Repeat 1 in annexin I contains the same valine for threonine substitution and loss of conserved acidic residue from the calcium binding site as repeat 1 of annexin II (see figure 1.1). Repeat 1 of annexins I and II are unlikely therefore to be able to bind Ca^{2+}, and may have acquired an alternative function. However, in another study the N-terminus (residues 1-45) of annexin I fused to the entire core domain of annexin V was sufficient to confer membrane aggregating ability on the chimera (Andree *et al.*, 1993). An alternative suggestion is that aggregation is not the result of protein-protein interactions but that a second phospholipid binding site is revealed after binding to the first membrane. If this is the case, the annexin I N-terminal domain may induce a global conformational change in annexin V that allows this to occur.

In vitro differences in the phospholipid-binding affinities of the different annexins are reflected in their interactions with biological membranes. Annexin VII aggregates and fuses rat pancreatic zymogen granules (requiring at least 100μM Ca^{2+}) and rat adrenal chromaffin granules (200μM Ca^{2+}) (Creutz *et al.*, 1992b). Monomeric annexin II requires mM Ca^{2+} to aggregate chromaffin granules, but the annexin II tetramer (see section 1.6) can aggregate chromaffin granule membranes at the physiologically relevant Ca^{2+} concentration of 1μM (Drust and Creutz, 1988). The tetramer inhibits aggregation of zymogen granules by annexin VII. Annexin VI binds to both types of organelle but is unable to cause aggregation, and in fact interferes with annexin II-mediated aggregation (Creutz *et al.*, 1992b). Annexin I also requires approximately 200μM Ca^{2+} for half-maximal aggregation of chromaffin granules (Wang and Creutz, 1992). Annexin I, abundant in the cytosol of neutrophils, can fuse neutrophil granules to phospholipid vesicles at 100μM Ca^{2+} (Meers *et al.*, 1992; Meers *et al.*, 1993). Interestingly annexin I requires higher concentrations of calcium to aggregate pure granules, and this requirement is lowered by treatment of the granules with trypsin (Meers *et al.*, 1993). This implies not only that the composition of the membrane is important but that proteins on the granule surface are involved, perhaps by shielding the phospholipid or by directly interacting with the annexin.

The calcium concentrations required for vesicle aggregation by most annexins *in vitro* are out of the physiological range (typically 0.1μM in a resting cell rising to 1-10μM in a stimulated cell), except for tetrameric annexin II. However recent reports of Ca^{2+} concentrations in the 100μM range in excitable cells immediately adjacent to the Ca^{2+}-channels in the plasma membrane and internal calcium stores just as they open, make it plausible that other annexins are involved in intracellular membrane fusion *in vivo* (Augustine and Neher, 1992).

1.3.3 Calcium-independent phospholipid binding

Although annexins are widely known as calcium-dependent phospholipid binding proteins, there are, in some cells and tissues, non-EGTA extractable pools of several different annexins. An "integral" membrane form of annexin I has been isolated from placenta (Sheets *et al.*, 1987); EGF-dependent phosphorylation converts it to a calcium-dependent binding form by an unknown mechanism. T51B rat liver epithelial cells also contain a calcium-independent bound form of annexin I (Campos-Gonzalez *et al.*, 1989). Annexins I and II remain associated with multivesicular bodies and early endosomal membranes respectively when treated with EGTA (Emans *et al.*, 1993, Futter *et al.*, 1993). Annexin VI and both isoforms of annexin V have been found in EGTA-resistant, detergent soluble forms in several different tissues, and calcium-independent membrane-bound forms of annexins IV and VII have also been reported (Bianchi *et al.*, 1992a, and references therein). It is not known how these attachments are achieved or whether in all cases they are reversible, but for some annexins a post-translational modification could be responsible (see section 1.3.4). Annexin II is found *in vivo* in a heterotetrameric complex with a protein called p11 (see section 1.6). This complex binds to phospholipid membranes in the effective absence of calcium: at 10^{-8}M Ca^{2+}, 70-80% of the tetramer is bound to phosphatidyl-serine liposomes (Powell and Glenney, 1987).

Does calcium regulate the function of the annexins in these cases? Although these pools of annexins have dispensed with the need for calcium for membrane binding, they may still require it to function; the permanent membrane attachment may simply serve to position or locate the protein correctly. It is not known whether the

phospholipid binding domain faces towards or away from the membrane in the calcium-independent forms of annexins; attachment via the N-terminus would leave the C-terminal core available to interact with other membranes.

1.3.4 Post-translational modifications

Annexins are post-translationally modified in several ways. Phosphorylation of tyrosine, threonine and serine residues in the N-terminus of several annexins, both *in vitro* and *in vivo* is well documented (for example, Isacke *et al.*, 1986), and is widely presumed to regulate activity. Protein kinase C phosphorylates annexins I, II, III, IV and VI (Khanna *et al.*, 1986b; Weber *et al.*, 1987; Barnes *et al.*, 1991; Stoehr *et al.*, 1990; Learmonth *et al.*, 1992). Tyrosine phosphorylation of annexins I and II has already been mentioned (section 1.3). Both tyrosine and serine phosphorylation of annexin I, by the EGF-R and PKC respectively, slightly reduce the calcium requirement for phospholipid binding but strongly inhibit its vesicle aggregating ability (Ando *et al.*, 1989; Wang and Creutz, 1992). Tyrosine phosphorylation of annexin II by $p60^{v\text{-}src}$ decreases the phospholipid binding-affinity of both the monomer and the tetramer (Powell and Glenney, 1987); phosphorylation of annexin II tetramer by PKC inhibits its ability to aggregate lipid vesicles (Johnstone *et al.*, 1992). Phosphorylation therefore may be an *in vivo* control mechanism for some annexins.

Several annexins including annexin II are acetylated at their N-termini. In the case of annexin II, the acetyl group is known to be an important part of the binding site for its cellular ligand p11 (Becker *et al.*, 1990). Goulet *et al.* (1992) report that annexins I and II are glycosylated. The intestinal-specific human annexin (ISA) is N-myristoylated, and probably associates with membranes through this lipophilic moiety (Wice and Gordon, 1992). Possibly the most intriguing post-translational modification is that of annexin VI. Annexin VI is phosphorylated in Swiss 3T3 fibroblasts and human T-lymphoblasts several hours after stimulation with growth factors. 2-D phosphoamino acid analysis reveals low levels of phosphorylation on serine and threonine but most of the phosphate is incorporated into an as yet unidentified complex termed phospho-X (plus a trace of a second unknown: phospho-Z) (Moss *et al.*, 1992). So far the precise

nature of phospho-X has eluded identification. It is released by acid hydrolysis within 20 minutes suggesting that it is not a phosphoamino acid, and it is attached not at the N-terminus but in the C-terminal half of the molecule (Moss and Jacob, personal communication).

1.4 Putative functions of the annexin family

Many functions have been proposed for annexin family members, generally based on circumstantial evidence and *in vitro* properties, but none has been unequivocally proven or universally accepted.

Roles in membrane trafficking have been postulated on the basis of their membrane-aggregating abilities, and are discussed further in sections 1.4.3 and 1.8.1 (Creutz, 1992; Burgoyne and Clague, 1994, for reviews). Possible involvement in cell growth control is suggested for annexins I and II since annexin I is phosphorylated by the EGF-R, and annexin II is phosphorylated on tyrosine by several transforming oncogenes, and in response to some growth factors (Isacke *et al.*, 1986). More specifically: annexin VI has been shown to act as a Ca^{2+}-dependent regulator of the Ca^{2+}-release channel of sarcoplasmic reticulum in artificial bilayers (Díaz-Muñoz *et al.*, 1990); "Placental anticoagulant protein-I" (annexin V) shows effective anti-coagulant activity *in vitro* (Reutelingsperger *et al.*, 1985), by coating membranes thus preventing coagulation factors from binding (Andree *et al.*, 1992) - however circulating levels of annexin V in plasma are so low that this is unlikely to be a genuine function (Flaherty *et al.*, 1990); annexin V, isolated as the collagen-binding protein anchorin CII, has been implicated in cartilage and bone calcification (Kirsch and Pfaffle, 1992); annexin V is also reported to be a specific protein kinase C inhibitor (Schlaepfer *et al.*, 1992c); annexin III was reported to be inositol 1,2-cyclic phosphate 2-phosphohydrolase (Ross *et al.*, 1990) and could therefore play a part in regulating cellular levels of inositol cyclic phosphates. Several annexins have been localised to the nucleus: annexin XI by Mizutani *et al.* (1992), annexin I by Raynal *et al.* (1992) and annexin II by Kumble and Vishwanatha (1991), where the latter is reported to be one of the primer recognition

proteins (PRPs) - accessory proteins for DNA polymerase-α in lagging strand DNA synthesis (Vishwanatha and Kumble, 1993).

Some of the more studied alternatives are described in detail below.

1.4.1 Annexins as calcium channels

Both annexins V and VII have been shown to display voltage-gated calcium channel activity in artificial membranes (Pollard and Rojas, 1988; Rojas *et al.*, 1990; Pollard *et al.*, 1992). The proposed mechanism for their channel activity is unlike that of other ion channels which are all membrane-spanning proteins. It has been suggested that in the calcium-bound state, the electrostatic potential gradient on the surface of annexin V in contact with the membrane is sufficient to create a membrane pore by electroporation (Karshikov *et al.*, 1992). Selectivity for calcium is imposed by the centrally located hydrophilic channel that runs through the annexin protein itself, and through which the ions are conjectured to pass. The salt bridges at opposing faces of the protein channel are proposed to gate the channel, and the small hinged domain motion could reflect open and closed states (Concha *et al.*, 1993). The two low affinity Ca^{2+}-binding sites in repeat 1, located at the bottom of the putative membrane pore in the region of highest negative potential, could be transient sites for calcium ions entering through the membrane. However, the relevance of the *in vitro* studies remains in doubt as this activity has yet to be demonstrated in whole cells.

1.4.2 Annexins as phospholipase inhibitors and anti-inflammatory agents

One of the first contexts in which annexins were discovered was as phospholipase A_2 (PLA_2) inhibitors (Huang *et al.*, 1986). All annexins demonstrate this property but the relevance of PLA_2 inhibition is equivocal since it is now generally accepted that this *in vitro* property is a consequence of calcium-dependent substrate screening rather than a direct effect on the enzyme itself (Davidson and Dennis, 1989). Indeed annexins have been shown to inhibit other phospholipases, including phospholipase C, presumably by the same mechanism (Machoczek *et al.*, 1989).

In vivo, PLA_2 produces arachidonic acid, the synthetic precursor of the inflammatory mediators leukotrienes and prostaglandins. This led to the idea that annexins could be involved in the glucocorticoid-mediated anti-inflammatory response. Although the case for this is weakened by the non-specific nature of the inhibition, glucocorticoid hormones are well established anti-inflammatory and immuno-suppressive agents and expression of some members of the annexin family, in particular annexins I and II, is induced in leukocytes by physiological levels of glucocorticoids (Peers *et al.*, 1993). In addition to this, the annexin I gene has putative glucocorticoid response elements in its promoter (Horseman, 1992).

In contrast to work that attributes the anti-inflammatory activity of annexins to the phospholipid binding activity of the core domains, is a study showing that an acetylated peptide of residues 2-26 of annexin I has strong anti-inflammatory activity of its own in several models of acute inflammation, although it is considerably less potent than the whole protein (Cirino *et al.*, 1993). These observations coupled with the recent report of specific annexin I binding molecules, "receptors", on the surface of peripheral blood monocytes and neutrophils (PMNs) (Goulding *et al.*, 1990), mean that annexin I at least, may have anti-inflammatory properties that are unconnected with its *in vitro* action as a PLA_2 inhibitor. Thus it is proposed that annexin I generated at sites of inflammation in response to raised glucocorticoid levels, binds to a cognate receptor on PMNs and down-regulates their phagocytic functions (Goulding and Guyre, 1992; Goulding and Guyre, 1993). There is circumstantial clinical evidence supporting a role for annexin I in down-regulating the immune response: some autoimmune disease patients produce anti-annexin I antibodies (Goulding *et al.*, 1989); some rheumatoid arthritis patients display greatly reduced numbers of these annexin I receptor proteins on the surface of their PMNs (Goulding *et al.*, 1992); and injection of annexin I into the intra cerebroventricles of rats reduces the infarct size and oedema caused by cerebral ischemia, while anti-annexin I antibodies increase the damage (Relton *et al.*, 1991). Furthermore antiserum to the N-terminal half of annexin I prevents the anti-inflammatory effects of a local injection of dexamethasone (Duncan *et al.*, 1993).

1.4.3 Annexins and endocytosis

In many cells endocytosis is a calcium-independent process. Pools of calcium-independent membrane-attached annexins which exist (see section 1.3.3) could be post-translationally adapted to function in endocytosis. Annexins I and II have both been localised to endosomal vesicles and there is evidence to suggest that they are involved in endocytosis. Annexin II is present on the cytoplasmic surface of early endosomal vesicles *in vivo*, and in an *in vitro* fusion assay, it and several other proteins are efficiently transferred from a pool of metabolically-labelled early endosomes to early endosomes immuno-isolated on beads (Emans *et al.*, 1993). In addition to this an expression plasmid containing the N-terminus of annexin II fused to its cellular ligand, p11, transfected into cells alters early endosome distribution (Harder and Gerke, 1993).

Annexin I may be involved at a much later stage of endocytosis. Internalised EGF-receptors (EGF-R) are sorted into vesicles in the multivesicular bodies (MVB). In NIH 3T3 cells transfected with normal or kinase-defective EGF-Rs, both functional and non-functional EGF-Rs are internalised after EGF stimulation. However, only EGF-Rs with a functional kinase are transferred to the internal vesicles of the MVB. Annexin I is not phosphorylated by EGF-R in the plasma membrane fraction of EGF stimulated cells, but in the MBV fraction, apart from the receptor itself, annexin I is the only protein phosphorylated by the EGF-R (Futter *et al.*, 1993). Annexin I is found attached to the plasma membrane and MVBs in the absence of calcium. *In vitro* phosphorylation by the EGF-R causes annexin I to become dependent on calcium for membrane association; and in cells with an active EGF-R there is less calcium-independent annexin I, suggesting that this occurs *in vivo* as well. This work suggests that annexin I could be the sensor for internalised EGF-R and mediate its inclusion in vesicles inside the MVB.

It has been suggested that annexin VI is necessary for the budding of clathrin-coated pits from the plasma membrane (Lin *et al.*, 1992). Brain cytosol depleted of annexin VI can no longer support pit budding. However, this has been disputed as not only did the assay require 100μM Ca^{2+}, but A431 cells, which have been extensively characterised as a model for the early steps of endocytosis, do not express annexin VI

mRNA (Smythe *et al.*, 1994). It is therefore unlikely that annexin VI is generally involved in the budding of clathrin-coated pits.

Genomic sequences coding for the C-terminal core domain of the annexin family appear to have undergone serial duplication events to produce the large family we know of today (Smith and Moss, 1994). The C-terminal domains are so well conserved within the family, and many properties are shared by the family members, that the possibility of functional redundancy is raised. The C-terminal domains may perform similar functions with the variable N-termini responsible for intracellular targeting and/or regulation of the core domain. Deletion of the annexin VII gene from *Dictyostelium* is not lethal and does not appear to impair development (Doring *et al.*, 1991). If other annexins are present in *Dictyostelium* then it is possible that some overlap of function between them allows the organism to survive. Otherwise it seems that the function of annexins is not an essential one to the survival of this organism.

The existence of annexins in lower eukaryotic phyla suggests that they have more basic functions than some of those currently being considered. If some of the specialised functions mentioned above, such as in bone calcification and anti-inflammatory roles, are indeed performed by annexins in mammals then they must have diverged from common ancestral annexins found in primitive organisms that do not require these functions. Annexins could have been adapted to these roles by the addition of N-terminal exons and further divergence, after gene duplication brought them into existence. As yet no one- or two-repeat annexins - the predicted ancestors of the four- and eight-repeat annexins - have been found, either in higher or lower eukaryotes.

One of the more plausible functions suggested for any member of the annexin family is the involvement of annexin II in exocytosis. The rest of the introduction will concentrate on annexin II and the cell system in which its possible function will be investigated - the rat basophilic leukaemia cell line, RBL-2H3.

1.5 Annexin II

1.5.1 Structure and calcium-binding

Annexin II is commonly called calpactin I because of its *in vitro* properties of binding to calcium and actin, and the fact that it is phosphorylated. The C-terminal core domain with four copies of the annexin repeat is attached to an N-terminus of 29 amino acids which is not homologous to any of the other annexins. The N-terminus is believed to regulate the activity of the protein as it contains all the known phosphorylation sites and the binding site for annexin II's cellular ligand, p11 (figure 1.2).

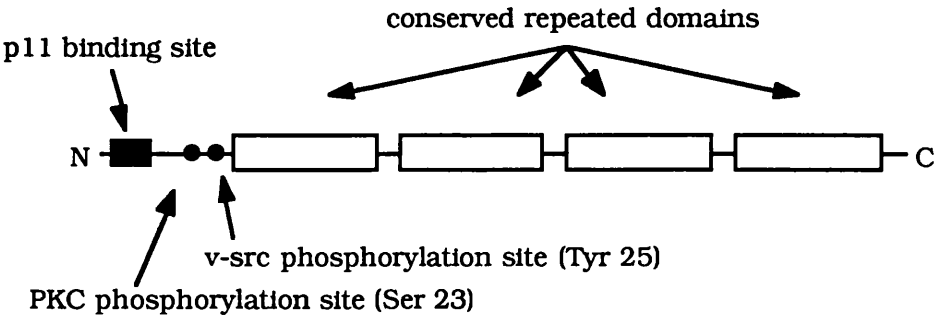


Figure 1.2 *The domain structure of annexin II*
The 70-amino acid repeated domains are represented by white boxes; the p11 binding site by a black box; and residues that are phosphorylation sites *in vivo* by circles.

Various experimental data support the assumption that the tertiary structure of annexin II is very similar to that of annexin V for which the crystal structure is known. Thiel *et al.* (1991a) determined that residues 27, 62, 65 and 67 of annexin II are essential parts of an epitope recognised by a particular monoclonal antibody; the equivalent residues lie close to each other in the annexin V crystal structure. The same group applied site directed mutagenesis to pinpoint residues involved in binding calcium; their results agreed with structural predictions (Thiel *et al.*, 1991b).

Only repeats 2 and 4 of annexin II contain the complete consensus sequence for a functional calcium-binding site as defined by the annexin V structure. Repeat 3, like repeat 3 of annexin V, has the second glycine and adjacent threonine residues followed

by an acidic residue 40 residues downstream so may still be able to bind calcium, although this was not observed in annexin V (see figure 1.1). Only one glycine remains of the consensus sequence in repeat 1 and therefore this site is not expected to be able to bind calcium. Repeat 1 does contain glutamic acid residues equivalent to those in annexin V which provide co-ordination points for the two low affinity calcium-binding sites.

Site-directed mutagenesis of the conserved acidic residue in each of repeats 2, 3 and 4 reduces the calcium affinity of the protein and increases the calcium requirement for phospholipid binding (Jost *et al.*, 1992). A cyanogen bromide-cleavage fragment of annexin II comprising the whole of repeat 1 will bind to phospholipid with an increased calcium requirement compared to the whole protein (Johnsson and Weber, 1990b). These last two results suggest that optimal spatial arrangement for phospholipid binding is achieved only when all 4 repeats are present, with all functional Ca^{2+}-binding sites intact. This precise spatial requirement could determine the specificity for binding to particular phospholipid head groups.

1.5.2 Annexin II phosphorylation on tyrosine

The initial interest in annexin II was due to its discovery as one of the main targets for tyrosine phosphorylation by the $pp60^{v\text{-}src}$ transforming tyrosine kinase. It is now known to be phosphorylated by the oncogenic products of several transforming viruses on tyrosine-23 in the N-terminus.

PDGF-stimulation of Swiss 3T3 cells results in tyrosine phosphorylation of several proteins including annexin II (Brambilla *et al.*, 1991). The presence of ortho-vanadate (a phosphotyrosyl phosphatase inhibitor) is required to detect this transient phosphorylation in intact cells indicating a rapid turnover. At low temperatures there is a lag before annexin II becomes phosphorylated which suggests that it is not a direct substrate of the PDGF-receptor. Annexins I and II are phosphorylated by the insulin receptor in rat liver cells (Karasik *et al.*, 1988).

The role of tyrosine phosphorylation of annexin II *in vivo* can only be guessed at. Phosphorylation of annexin II by $pp60^{v\text{-}src}$ decreases its binding to phospholipid

vesicles at all Ca^{2+} concentrations, but does not prevent it binding to p11 (Powell and Glenney, 1987). A comparison of normal and RSV-transformed chick fibroblasts found that the majority of annexin II was associated with the cytoskeleton in both, which probably indicates that tyrosine phosphorylation does not affect this association (Zokas and Glenney, 1987) (see section 1.6.3). The likelihood of functional significance is perhaps slightly diminished by the fact that annexin II from *Xenopus* while being 80% identical to the mammalian protein, has lost the conserved src phosphorylation site (tyrosine-23) (Izant and Bryson, 1991). Any control exercised by tyrosine phosphorylation on annexin II function would therefore be lost in *Xenopus*.

1.5.3 Annexin II phosphorylation on serine and threonine

The addition of PKC activators to some cell types results in an increase in serine phosphorylation of annexin II (Oudinet *et al.*, 1993), although the stoichiometry is low (Gould *et al.*, 1986). *In vitro*, PKC phosphorylates annexin II with a stoichiometry of approximately 1 mole of phosphate/mole of annexin II (Khanna *et al.*, 1986a). The proximity of bound p11 to the phosphorylation site of annexin II probably inhibits the phosphorylation reaction in the tetramer, although Johnstone *et al.* (1992) found under optimal conditions that 2.1+/-1.2 mole of phosphate were incorporated per annexin II tetramer. The N-terminal domain of annexin II is also phosphorylated on serine *in vitro* by CaM- and cAMP-dependent kinases. Monomeric annexin II phosphorylated by CaM- or cAMP-dependent kinases cannot associate with p11 (Johnsson *et al.*, 1986). Annexin II has two phosphorylation sites for PKC in the N-terminus, one of which is serine-25. Phosphorylation on this serine does not prevent annexin II associating with p11 to form the tetramer, nor does it affect the ability of the annexin II tetramer to bind to vesicles. PKC phosphorylation does however decrease the ability of the tetramer to aggregate phospholipid vesicles (Johnstone *et al.* 1992). Thus, phosphorylation of serine-25 appears to prevent a Ca^{2+}-dependent conformational change that normally allows tetramer bound to one vesicle to interact with a second membrane and cause aggregation.

Of the four other serine residues in the N-terminal of annexin II (at positions 1,

11, 17 and 21), serine-11 most closely resembles a kinase consensus site, near a lysine and an arginine, and is therefore the most likely candidate for phosphorylation by CaM- and cAMP-dependent kinases and PKC (Schlaepfer and Haigler, 1988). Serine-11 is within the p11 binding site so phosphorylation at this site would be expected to interfere with p11 binding. cAMP-dependent kinase also produces a trace of phosphothreonine.

1.5.4 Annexin II interaction with the cytoskeleton

In vivo, the annexin II tetramer is generally associated with the cytoskeleton close to the inner leaflet of the plasma membrane (Osborn *et al.*, 1988). The cytoskeleton in non-erythroid cells is composed of, amongst other things, F-actin and the spectrin-related protein fodrin. The original studies of interaction between the annexin II_2p11_2 tetramer and both fodrin and actin required unphysiologically high calcium concentrations (mM) to demonstrate binding (Gerke and Weber, 1984; Glenney, 1986). However, the annexin II_2p11_2 tetramer has been shown to cause Ca^{2+}-dependent bundling of F-actin in μM Ca^{2+} (Ikebuchi and Waisman, 1990). A region of annexin II that is homologous to the actin-binding region of myosin, residues 286-294, is probably involved in F-actin bundling by annexin II. Pre-incubation of actin with a peptide of residues 286-294 of annexin II inhibits the bundling activity of the tetramer, and this can be overcome by excess tetramer (Jones *et al.*, 1992). Further addition of excess peptide does not reverse bundle formation, suggesting that the reaction is not reversible.

It has been suggested that annexin II could function like ankyrin in erythrocytes which attaches the plasma membrane to spectrin in the cytoskeleton. However, the interaction between annexin II and fodrin is non-specific and of low affinity (Cheney and Willard, 1989), and therefore unlikely to be important *in vivo*.

1.6 Annexin II and its interaction with p11

Annexin II is isolated from cells either as a monomer or as a 90kD tetrameric complex comprised of two molecules of annexin II and two of an 11kD protein, p11. The

relative proportions of monomer to tetramer vary - ranging from 50% tetramer in fibroblasts to almost 100% tetramer in intestinal epithelium (Zokas and Glenney, 1987). p11 dimerises spontaneously and readily forms the tetrameric complex independently of calcium. The tetramer is extremely stable - its dissociation constant is below 30nM (Johnsson *et al.*, 1988) and it requires urea or oxidation of -SH groups in p11 (kept in a reduced state in the intracellular environment) to dissociate.

1.6.1 p11, the cellular ligand of annexin II

p11 (also known as p10) is 97 amino acid residues in length and is a member of the S100 family of proteins. The first identified members of the family were isolated from brain tissue as the only proteins soluble in 100% $(NH_4)_2SO_4$ (w/v) (Dannies and Levine, 1971). They are small, dimeric proteins that characteristically have two EF-hand type Ca^{2+}-binding structures. Other members of the S100 family include calcyclin (expressed in a cell cycle dependent manner), which has been found associated with annexin XI (Tokumitsu *et al.*, 1992), and calgranulins (induced during inflammatory responses).

p11 is unique within the S100 family because, due to amino acid deletions or substitutions in both of its EF-hand loops, it cannot bind calcium. It is therefore presumed to be permanently activated and to function independently of calcium. A further difference between p11 and other S100 family members is that p11 has a C-terminal extension (residues 78-96) after the last helix of the second EF-hand. The 18 amino acids of the C-terminal are particularly highly conserved, and are therefore likely to be structurally and functionally important. It is through these C-terminal amino acid residues that p11 is believed to interact with annexin II (see section 1.6.5).

p11 has been cloned from a variety of species: mouse and cow (Saris *et al.*, 1987), rat (Masiakowski and Shooter, 1988), pig (Gerke and Weber, 1985), human, chicken and toad (Dooley *et al.*, 1992, Kube *et al.*, 1991). Mammalian and avian p11 share approximately 90% identity, *Xenopus laevis* shares only 60% amino acid identity with both, although the C-terminal 18 residues are more highly conserved. Oddly, the human protein is 100% identical to the ungulate species (bovine and porcine), but only

91-92% identical to the rodents which are closer to humans in evolutionary terms.

Binding to p11 alters some of the properties of annexin II. The tetramer has a greatly enhanced affinity for calcium, binding to phosphatidylserine liposomes in the effective absence (10^{-8}M) of Ca^{2+} (Powell and Glenney, 1987), and so membrane attachment of the tetramer in the cell is unlikely to be controlled by calcium levels. Annexin II undergoes a conformational change when both Ca^{2+} and phospholipid are bound; this change is greater when annexin II is complexed with p11 (Follenius-Wund *et al.*, 1993). The increased conformational change could explain why the annexin II_2 $p11_2$ complex is able to aggregate chromaffin cell granules at physiological calcium concentrations, whereas monomeric annexin II can only bind to them. p11 binding does not change the binding preference for phospholipid (PA>PS>PI).

PKC phosphorylation of annexin II is reduced when p11 is bound (Khanna *et al.*, 1986a), and as mentioned in section 1.5.3, phosphorylation of annexin II can prevent it associating with p11. Does p11 have a role in regulating phosphorylation of annexin II, or, does phosphorylation regulate p11 association with annexin II? There are distinct pools of monomeric and tetrameric annexin II *in vivo* which suggests several possibilities: phosphorylation works in conjunction with p11, i.e. the tetramer is phosphorylated as a switch off or on mechanism; phosphorylation of annexin II is used to prevent it associating with p11; or p11 protects annexin II from phosphorylation.

1.6.2 Expression of annexin II and p11

Annexin II has been identified as the growth-regulated gene IB6 (Keutzer and Hirschhorn, 1990), and has been shown to be cell cycle regulated in CHO cells (protein and mRNA levels peak as the cells move from G1 to S-phase (Chiang *et al.*, 1993) which could relate to its proposed role in DNA replication (Vishwanatha and Kumble, 1993)). Several studies on the expression of annexin II and p11, protein and mRNA, have been reported (Gerke, 1989b for a review). Zokas and Glenney (1987) concluded that p11 and annexin II proteins were co-ordinately expressed since in all the bovine tissues they investigated they were present at similar levels, and neither was found in the absence of the other. Osborn *et al.* (1988) investigated the tissue distribution of p11 and annexin II

in various porcine and bovine tissues and found identical distribution of staining for p11 and annexin II. Cardiac, smooth and striated muscle, neuronal cells, hepatocytes and cortical tubular cells of the kidney were negative for both, while epithelial cells, fibroblasts, blood vessels, Schwann cells, non-muscle cells of the heart and brain endothelial cells contained both, as did all the cultured cell lines these authors investigated.

This is not always the case though: in F9 teratocarcinoma cells p11 is expressed fairly constantly, whereas annexin II expression is normally undetectable but is strongly increased when the cells are induced to differentiate by retinoic acid or by elevation of cyclic AMP (Harder *et al.*,1993). Before differentiation, p11 is found throughout the cytoplasm, but upon annexin II expression p11 is recruited to the sub-membraneous region of the cell. While p11 may have a function on its own, this expression of p11 in the absence of annexin II may be an oddity of this particular (transformed) cell line. Saris *et al.* (1987) also suggested that p11 and annexin II mRNA are not always co-ordinately expressed. Murine brain and embryonic cell lines have high levels of p11 mRNA but very low amounts of annexin II mRNA. Conversely heart and testis express reasonable amounts of annexin II mRNA but extremely low or undetectable levels of p11 mRNA. However, mRNA levels may not accurately reflect protein levels in cells as the turnover of the protein must also be considered.

The two proteins are certainly coregulated in some cell types: Fox *et al.* (1991) found that p11 and annexin II were both induced to varying degrees in PC12 cells within hours of treatment with nerve growth factor or epidermal growth factor. Pulse-chase analysis of annexin II and p11 in human fibroblasts revealed that the turnover of the cytoskeletal pool of annexin II was much slower (a half-life of 40-50 hours) than the soluble pool (half-life of 15 hours) (Zokas and Glenney, 1987). p11 turned over at approximately the same rate as the cytoskeleton-associated annexin II. This indicates that the two pools of annexin II are not freely interchangeable, and that once attached to the cytoskeleton the tetramer is not released. The tetramer is presumably in place to perform its cellular function (if attachment of p11 was a prelude to destruction the tetramer would have a shorter half life than monomeric annexin II). Binding to p11 is

not a reversible regulatory step since the tetramer is extremely stable. Instead, binding p11 appears to be an enabling step which recruits annexin II to its site of action. Monomeric annexin II may have a different role in the cell since it has different properties and location from the tetramer. This could be partially regulated by other S-100 proteins once all available p11 has formed the heterotetramer; for example S-100 from bovine brain binds to annexin II monomer *in vitro* (Bianchi *et al.*, 1992b). The binding affinity of S100 for annexin II is an order of magnitude lower than that of p11, and binding is rapidly reversed when the Ca^{2+} concentration falls. This interaction may not be specific since S100 proteins are known to be "sticky", and it is certainly unexpected since the binding site of p11 for annexin II is in its C-terminal 18 residues, which are not homologous with the rest of the family. However, S-100 is sufficiently abundant in the cytoplasm of some cells to raise the possibility of interaction with monomeric annexin II when the calcium levels rise.

1.6.3 Cellular localisation of annexin II and dependency on tetramer formation

When cells are permeabilised in the presence of a cytoskeletal stabilising buffer (containing potassium ions) a characteristic reticular network is stained by antibodies raised against fodrin or actin. A similar pattern of staining is generated by antibodies directed against annexin II or p11 (Greenberg and Edelman, 1983; Zokas and Glenney, 1987). p11 is located exclusively in the sub-membrane area in tight association with annexin II (Zokas and Glenney, 1987; Osborn *et al.*, 1988). The staining of annexin II is more diffuse, presumably reflecting the fact that monomeric annexin II is also present in the cytosol. Annexin II is absent from areas of actin-containing stress fibres in the cell.

Annexin II absolutely requires p11 binding (and vice versa) to form a stable association with the sub-membraneous cytoskeleton. Mutation of either of the isoleucine or leucine residues (positions 6 and 7) in annexin II (see section 1.6.4) to glutamic acid reduces the binding of p11 to annexin II to undetectable levels (Thiel *et al.*, 1992). Immunofluorescent staining of HeLa cells transiently transfected with such a

p11 binding-incompetent form of annexin II revealed that the heterologous annexin II was not located in the characteristic reticular network but distributed evenly throughout the cell. Normal annexin II microinjected into HeLa cells becomes attached to the cytoskeleton within 3 hours (Osborn *et al.*, 1988). Since half-life data show that the tetramer does not exchange with the cytosolic pool, the microinjected annexin II probably combines with newly synthesised p11. Osborn *et al.* (1988) suggest that there are free "receptors" for annexin II (or the tetramer) on the cytoskeleton but do not explain why the endogenous monomeric annexin II is not bound there.

A small proportion of p11 injected into rat mammary RMCD cells becomes incorporated into the cytoskeletal network; a p11 derivative carboxymethylated on cysteine-82, that is unable to bind annexin II, does not become associated with the sub-membraneous cytoskeleton when injected into RMCD cells (Osborn *et al.*,1988). Therefore p11 is thought to require annexin II to bind to the cytoskeleton. The annexin II_2p11_2 complex apparently also requires calcium for cytoskeleton association since an annexin II mutant with defective Ca^{2+}-binding sites (substitution of the conserved acidic amino acid in each of the three high-affinity calcium-binding sites) remains cytosolic in transfected HeLa cells (Thiel *et al.*, 1992). p11 enhances the affinity of annexin II for calcium which could be a crucial factor for cytoskeleton binding at the resting calcium concentration in the cell.

1.6.4 The binding site for p11 on annexin II

A thorough study of single residue substitutions in a 14 amino acid peptide of the N-terminal of annexin II by Becker *et al.*, (1990) confirmed that the binding site for p11 consists of the first 12 residues and the N-acetyl group of serine-1.

Ac-S T V H E I L C K L S L . . .

This part of the protein forms an amphipathic helix. The most important contributors to the binding energy are the non-polar residues valine-3, isoleucine-6, leucines-7 and -10, and the N-acetyl group. These hydrophobic residues are predicted to line up along one side of this helix (see figure 1.3). Replacing any of these with a charged residue virtually abolishes p11 binding. Substitutions of residues on the

hydrophilic face have much less effect, unless they are replaced by proline, which is known to interrupt helical backbones and therefore probably alters the conformation of the binding domain substantially. Attachment of the fluorophore Prodan (6-propionyl-2-dimethylamino-naphthalene) to cysteine-8 of annexin II does not prevent p11 binding (Johnsson *et al.*, 1988), which also implies that cysteine-8 (on the hydrophilic face) is not in contact with p11.

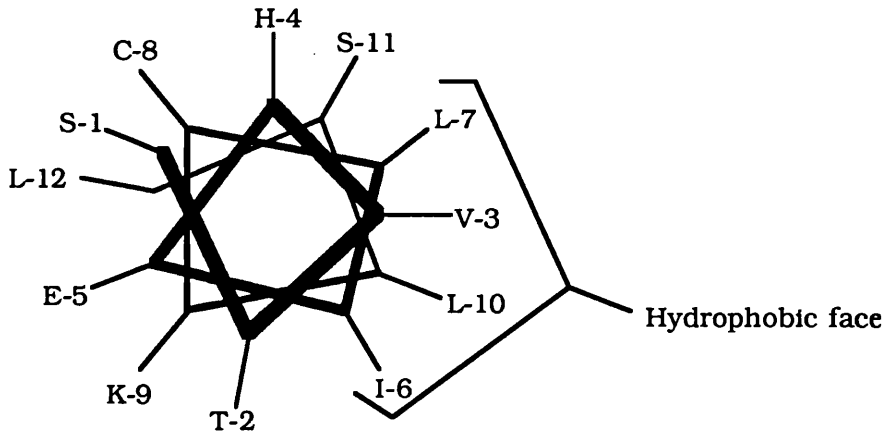


Figure 1.3 *Representation of the helical conformation of the region of annexin II that binds to p11.*
Adapted from Becker *et al.* (1990). Amino acids are represented by the single letter amino acid code, followed by their residue number in the annexin II sequence.

The surface of the binding site probably fits fairly precisely to the p11 surface since substitution of the hydrophobic residues 3, 6 and 7 with other hydrophobic residues of different size causes significant loss of binding affinity. Theoretical calculations are consistent with the hydrophobic face of the helix becoming buried in the interior of the protein complex on tetramer formation (Becker *et al.*, 1990).

The p11 binding domain of annexin II is 100% conserved in mammals; chicken annexin II has one substitution on the hydrophilic surface, cysteine-8 is replaced by serine. The p11-binding region of *Xenopus* annexin II has 4 amino acid substitutions including valine-3 for the also hydrophobic residue isoleucine (Izant and Bryson, 1991). Substitution of a contact residue is probably accommodated by changes in the binding site for annexin II on p11 to maintain a precise fit.

The p11 binding domain of annexin II, unique amongst the annexin family, is contained within a single exon, separate from those encoding the core domain. In this light it is interesting that a cell line transfected with c-*raf-1* that became transformed was found to have spliced the p11 binding exon of annexin II onto the kinase domain of c-*raf-1* (Mitsunobu *et al.*, 1989). This is presumably akin to how annexin II diverged from a common ancestral annexin - by attachment of the p11 binding domain to the conserved core domain - and indicates that this domain can have profound consequences on the activity of the protein to which it is attached.

The only other established interaction between an annexin and another protein also involves a member of the S100 family. Annexin XI (CAP-50) binds to calcyclin. Annexin XI has an extremely long, relatively hydrophobic N-terminal domain of almost 200 amino acid residues. Systematic examination of N-terminal deletion mutants has narrowed down the binding site for calcyclin to the first 52 residues of annexin XI (Tokumitsu *et al.*, 1993). Of these 35 are hydrophobic. There does not appear to be the capacity for amphipathic helix formation (there are 18 proline residues) in this stretch. So while hydrophobic interactions could well be important for calcyclin binding to annexin XI, the structural motifs that mediate annexin II binding to p11 are likely to be very different.

1.6.5 The binding site for annexin II on p11

Kube *et al.* (1992) produced several mutants of p11 truncated within the C-terminal extension, and assessed them for their ability to bind to annexin II. They deduced that a highly hydrophobic region of p11, residues 85 to 91, were essential for annexin II binding. Three residues with large hydrophobic side chains were identified as particularly important, tyrosine-85, phenylalanine-86 and methionine-90. These may well be in direct contact with the hydrophobic site identified on annexin II. Mutation of valine-87 to alanine also reduced binding somewhat (valine-88 was not investigated because it is not conserved in *Xenopus*).

Mutation of cysteine-82 of p11 to glutamine completely abolished binding. The reason for this is not clear: steric hindrance is unlikely since substitution by serine

(which has similar dimensions to cysteine) also produces a drastic effect. This suggests that there is a specific requirement for the particular charge distribution around the cysteine side chain. Alkylation of cysteine-82 prevents tetramer formation, and this cysteine is protected from alkylation in the tetramer (Johnsson and Weber, 1990a), which implies that cysteine-82 is in contact with annexin II in the tetramer.

Interestingly, a p11 C-terminal peptide of residues 78-96 interacted only very weakly in binding studies with annexin II (Becker *et al.*, 1990). Presumably the C-terminal residues require the rest of the protein to present a properly folded site for annexin II binding. (A crystal structure of p11 would be necessary to establish this.) This is unlike annexin II in which the N-terminal domain folds independently of the rest of the protein: in solution it has an ill-defined structure but takes on an α-helical conformation on binding p11 (Johnsson *et al.*, 1988).

1.7 Secretion from rat basophilic leukaemia (RBL-2H3) cells

A rat basophilic leukaemia cell line, RBL-2H3, is routinely used as a model for mast and basophil cells active in the acute allergic reaction. Cross-linking of high affinity IgE receptors (FcεR1) on the surface of these cells by multivalent antigen (Metzger, 1992) leads to the release of histamine and other inflammatory mediators, including arachidonic acid metabolites (Scheimer *et al.*, 1986). Release of these mediators occurs in two separate ways: degranulation, where pre-synthesised compounds such as serotonin and histamine are released from granules by exocytosis; and *de novo* synthesis of leukotrienes and prostaglandins.

The high affinity IgE receptor, FcεR1, on the surface of mast cells, basophils and RBL-2H3 cells is a heterotetramer consisting of four polypeptide chains ($\alpha\beta\gamma_2$). The mechanism of signal transduction by the receptor is not yet known. The receptor is not a tyrosine kinase itself but cross-linking the receptor does result in tyrosine phosphorylation of several cellular proteins (Benhamou *et al.*, 1990) including subunits of the receptor and phospholipase Cγ1 (Li *et al.*, 1992). In RBL-2H3 cells it is reported that $p56^{lyn}$ and $pp60^{src}$ (and $p62^{yes}$ in a mouse mast cell line), members of the *src* family of cytoplasmic tyrosine kinases, are activated after cross linking FcεR1, and that $p56^{lyn}$

coprecipitates with FcεR1 (Eiseman and Bolen, 1992). Tyrosine phosphorylation occurs within 30 seconds of stimulation and persists for up to 30 minutes which correlates with the time course of secretion.

The response to stimulation in RBL-2H3 cells involves metabolism of inositol phospholipids (Beaven *et al.*, 1984) and a transient rise in intracellular calcium concentration (Millard *et al.*, 1989). It is currently believed that phospholipase Cγ1 (PLCγ1) once activated, probably by tyrosine phosphorylation of the enzyme in response to FcεR1 cross-linking (Li *et al.*, 1992), hydrolyses phosphatidylinositol-4,5 bisphosphate (PIP_2) to produce inositol-1,4,5 trisphosphate (IP_3) and diacylglycerol (DAG), which raise intracellular calcium concentrations and activate PKC. Activation of PKC through binding DAG and Ca^{2+} results in phosphorylation of membrane-bound and cytosolic target proteins. Phosphatidylcholine (PC) hydrolysis appears to be regulated by PKC which is at least partially due to the action of PC-PLC (which hydrolyses PC directly to DAG) (Nakashima *et al.*, 1991). Thus the biphasic increase in DAG reported in RBL-2H3 cells may be the result of the initial PIP_2 hydrolysis causing PKC activation which in turn leads to PC hydrolysis and further DAG production. Evidence is accumulating that phospholipase D (PLD) is also activated when RBL-2H3 cells are stimulated (Lin *et al.*, 1991, and references therein). PLD hydrolyses PC to phosphatidic acid (PA), which is subsequently hydrolysed to DAG, and choline. Thus PLD may have an important part to play in production of DAG and arachidonic acid (AA), the precursor of leukotrienes and prostaglandins. The effect of PLD on the PI:PC ratio in vesicle membranes, and the production of PA, may influence membrane fusion. It has recently been shown that PLD is regulated by ARF (Cockcroft *et al.*, 1994) - a GTP-binding protein known to be involved in vesicle trafficking (Balch, 1990).

There is evidence from other cell types that there is an absolute requirement for inositol phospholipids (and not their breakdown products) in the plasma membrane for exocytosis to occur. Depletion of PI in permeabilised chromaffin cells by a PI-specific bacterial PLC causes inhibition of secretion subsequently triggered by calcium (Eberhard *et al.*, 1990). This is not due to lack of DAG as that produced by the bacterial PLC persists during the period of stimulation by calcium, and inhibition is not relieved

by addition of exogenous IP_3. The importance of PI to membrane structure and properties is indicated by the recent discovery that a PI-transfer protein (PI-TP) that shuttles PI between membranes *in vitro* is necessary for secretion in PC12 cells (Hay and Martin, 1994). The presence of PI may be necessary for particular proteins to interact with the plasma membrane, for instance annexin II has a preference for binding to PI and PS. (However, in this system it cannot be ruled out that the PI requirement is for vesicle trafficking rather than exocytosis itself.)

Secretion from basophils appears to occur by a separate pathway in response to univalent stimuli, such as the bacterial peptide fMet-Leu-Phe or the complement peptide C5a, rather than to multivalent stimuli. Both stimuli produce a characteristic increase in intracellular calcium concentration, but various inhibitors of secretion have completely different effects on secretion induced by the two different types of stimuli. Pertussis toxin (which ADP-ribosylates some G-protein subunits of the α_1 and α_0 classes) completely inhibits secretion in response to fMet-Leu-Phe but at the same concentration has no effect on IgE-stimulated secretion from human basophils (Saito *et al.*, 1987), implying that a pertussis toxin-sensitive G-protein(s) is involved in the response to fMet-Leu-Phe but not to IgE. Similarly, mast cell secretion in response to mastoparan or compound 48/80 is inhibited by pertussis toxin, whereas the IgE-response is not (Saito *et al.*, 1987). Okadaic acid (an inhibitor of the cytosolic serine and threonine phosphatases types 1 and 2A) strongly inhibits IgE-mediated secretion from human basophils but has no effect on fMet-Leu-Phe-stimulated secretion (Botana and MacGlashan Jr., 1993). The pathway triggered by univalent ligands appears to have some other method of activating the protein(s) that, in the IgE response, require dephosphorylation (unless a completely different set of proteins is involved for exocytosis in the two pathways, which is unlikely).

1.7.1 The role of calcium in exocytosis

Studies in basophils and mast cells

It is generally accepted that a transient rise in intracellular calcium concentration can evoke exocytosis in many cell types. Basophils show a biphasic calcium

response to IgE-receptor cross-linking: the first peak lasts for 50 seconds, is not dependent on extracellular calcium and is not sufficient to cause degranulation; the later more sustained rise, involving extracellular calcium, is required for histamine release (MacGlashan and Botana, 1993). The average cytosolic values are similar for both phases of the calcium response which suggests that the exocytotic machinery experiences, and responds to, much higher calcium levels just under the plasma membrane as extracellular calcium enters.

Non-excitable cells, which do not possess voltage-dependent calcium channels, have two main mechanisms for calcium entry: a non-specific cation channel which is receptor- or second messenger-operated (known as the 50pS-channel in mast cells) (Fasolato *et al.*, 1993); and a highly selective hyperpolarisation-driven calcium current that is activated by depletion of cellular calcium, I_{CRAC} ($\underline{C}a^{2+}$ $\underline{r}$elease $\underline{a}$ctivated $\underline{c}$urrent) (Hoth and Penner, 1993). The calcium channel responsible for the influx of Ca^{2+} ions into RBL-2H3 cells in the first few minutes is thought to be a target for the anti-inflammatory drug cromoglycate, and a cromoglycate-binding protein (CBP) on the surface of RBL-2H3 cells has been proposed as the channel (Mazurek *et al.*, 1984; Ran and Rivnay, 1988). It is very different from the calcium channel in excitable cells as depolarisation inhibits calcium entry (and exocytosis) from RBL-2H3 cells (Mohr and Fewtrell, 1987).

The delay between stimulation and exocytosis in RBL-2H3 cells is much greater than for neuroendocrine cells. There is evidence that the rise in intracellular pH that follows cross-linking of the IgE receptor lowers the calcium concentration required for exocytosis (Ali *et al.*, 1989a) which could explain how exocytosis is maintained after the initially higher calcium levels have dropped.

Studies in chromaffin cells

There is also evidence that influx through the calcium channels, rather than raised average cytoplasmic calcium is necessary for exocytosis from chromaffin cells. Nicotinic stimulation of chromaffin cells causes membrane depolarisation, opening voltage-dependent calcium channels over the whole plasma membrane, and exocytosis then occurs over the entire cell surface (Cheek *et al.*, 1989). Angiotensin II is a weak

secretagogue that mobilises inositol 1,4,5-trisphosphate and so releases calcium from intracellular stores. Although treatment of chromaffin cells with angiotensin II generally results in similar average cytosolic calcium concentrations to nicotinic stimulation, most cells do not secrete in response to the release of calcium from intracellular stores. Secretion in response to angiotensin II is prevented by removing extracellular calcium and is therefore probably caused by local calcium influx (perhaps triggered by depletion of intracellular stores) (Cheek *et al.*, 1989).

There is a delay of between 5 and 100 milliseconds between depolarisation (and opening of voltage-dependent calcium channels) and fusion of the majority of vesicles in chromaffin cells (Chow *et al.*, 1992). Concentrations of Ca^{2+} up to 100μM are postulated to occur in the first few milliseconds after calcium channel opening (Augustine and Neher, 1992) with 5-30 μM Ca^{2+} persisting in the sub-membraneous compartment for perhaps the next 100 milliseconds being sufficient to support exocytosis. A steady rate of exocytosis can be achieved in the presence of barium (which enters through Ca^{2+}- channels but does not inactivate them and is probably not effectively buffered by the cell), rather than the transient exocytosis observed in the presence of calcium (Michelena *et al.*, 1993), suggesting that raised cytosolic calcium is sufficient to maintain exocytosis in chromaffin cells, although the initial Ca^{2+} peak may be necessary to trigger the process.

Work by Neher and Zucker (1993) in chromaffin cells and Thomas *et al.* (1993) in pituitary melanotrophs, found distinct kinetic components to secretion which they postulate could be due to different pools of vesicles, some very close to the plasma membrane which are released in the first few tens of milliseconds, others which are subsequently mobilised by calcium to reach the plasma membrane.

1.7.2 GTP binding-proteins and exocytosis

In addition to a rise in intracellular calcium concentration, permeabilised RBL-2H3 cells require ATP and activation of one or more GTP-binding proteins to trigger secretion (De Matteis *et al.*, 1991). Intact mast cells can be stimulated to secrete by mastoparan and compound 48/80 which activate guanine nucleotide exchange on

heterotrimeric GTP-binding proteins (known as G-proteins) in the plasma membrane (Aridor *et al.*, 1990). Part of this effect may be due to activation of a G-protein that controls PLC activity, but these agents can also stimulate secretion in the absence of phospholipid metabolism (using neomycin or high GTPγS to inhibit PLC). This implies that they can act at a G-protein downstream of PLC activation. The putative G-protein involved in the terminal stages of secretion has been dubbed G_E (Gomperts, 1990). In mast cells, G_{i3} has been identified as a pertussis toxin-sensitive heterotrimeric G-protein that activates exocytosis downstream of PLC activation (Aridor *et al.*, 1993) and therefore is a candidate for G_E. In permeabilised chromaffin cells, various GTP analogues have been shown to stimulate calcium-independent exocytosis through a GTP-binding protein distinct from that which activates PLA_2 (Morgan and Burgoyne, 1990). Work by Sontag *et al.* (1991) has also strongly implicated a pertussis toxin-sensitive G-protein in the later stages of secretion from chromaffin cells, after PKC activation and calcium entry. This G-protein however, has an inhibitory effect on exocytosis. The nature of communication between calcium and one or more G-proteins in the exocytotic pathway is unknown. However, since intracellular GTP concentrations do not vary significantly *in vivo*, calcium must enable guanine nucleotide binding proteins to respond to the levels of GTP present: it has been suggested that a calcium-binding protein (C_E) stimulates exchange of guanine nucleotides on G_E, by promoting release of GDP (Lillie and Gomperts, 1990).

Several small GTP binding-proteins of the ras superfamily have been implicated in exocytosis: rac and rho are thought to control cytoskeletal rearrangements during exocytosis (Norman *et al.*, 1994); down regulation of rab3B expression inhibits exocytosis from rat anterior pituitary cells (Lledo *et al.*, 1993), and a rab3A effector domain peptide stimulates exocytosis from patch-clamped mast cells (Oberhauser *et al.*, 1992). However, the effect of rab3 effector domain peptide is removed by pertussis toxin (Law *et al.*, 1993) suggesting an interaction with a pathway involving a trimeric G-protein.

1.7.3 The cytoskeleton and exocytosis

The sub-plasma membrane cortical cytoskeleton composed of actin and its regulatory proteins, presents a physical barrier to secretory granules in resting cells. For exocytosis to occur it is widely presumed that this barrier must rearrange to allow granules access to the plasma membrane. Agents that elicit exocytosis in RBL-2H3 cells (Pfeiffer *et al.*, 1985), chromaffin cells (Burgoyne *et al.*, 1989), mast cells (Koffer *et al.*, 1990) and parotid acinar cells (Perrin *et al.*, 1992) result in cortical F-actin disassembly. This decrease in the amount of F-actin in the cytoskeleton (i.e. Triton-X 100 insoluble material) correlates with a fragmentation of the cortical ring in chromaffin cells (Trifaró *et al.*, 1992, Burgoyne *et al.*, 1989). Evidence that this rearrangement is necessary for exocytosis includes the demonstration that agents which stabilise F-actin (phalloidin) inhibit secretion, while agents that destabilise actin filaments (cytochalasins B, D and E, DNase I) can enhance calcium-dependent secretion in RBL-2H3 cells (Narasimhan *et al.*, 1990), mast cells (Koffer *et al.*, 1990) and chromaffin cells (Lelkes *et al.*, 1986); and cytochalasin B treatment of neutrophils is necessary for exocytosis in response to fMet-Leu-Phe *in vitro* (Bennett *et al.*, 1980).

In mast cells stimulated to secrete, F-actin relocalises from the cortical cytoskeleton to the cell centre (Norman *et al.*, 1994, in press). However, electron microscopy and light microscopy studies of chromaffin cells found no evidence of wholesale reorganisation of the cortical cytoskeleton upon stimulation (Nakata and Hirokawa, 1992), rather a modest transient rearrangement with the cortical actin layer becoming very thin at localised sites of exocytosis, indicated by anti-dopamine β-hydroxylase-fluorescence (a protein of the granule inner membrane used as a marker for exocytosis). Cytoskeletal changes may be very different in different secretory cells depending on the extent of response required and the nature of the intracellular mediators. *In vitro*, in response to Ca^{2+} and GTPγS, permeabilised mast cells release their entire secretory granule population (Hide *et al.*, 1993) which may well require large-scale rearrangement of the cortical cytoskeleton, whereas chromaffin cells release only a percentage at a time, requiring more minor changes.

In RBL-2H3 cells, although IgE receptor cross-linking causes an initial decrease in cytoskeletally-attached F-actin, there is a rapid increase in the *total* F-actin content (Apgar, 1991). Mast cells also display an increase in total F-actin content after stimulation (Norman *et al.*, 1994, in press). This suggests that apart from dismantling the cortical actin barrier, actin polymerisation makes a positive contribution to exocytosis. Myosin phosphorylation by myosin light chain kinase (MLCK) is observed in RBL-2H3 cells stimulated through the IgE-receptor (Peleg *et al.*, 1992). Phosphorylation on serine-19 and threonine-18 by MLCK initiates the actin-activated MgATPase activity of myosin, which is consistent with an active role for the cytoskeleton during exocytosis in these cells.

Raising intracellular calcium causes cortical F-actin disassembly in permeabilised mast cells and chromaffin cells, however actin disassembly may be calcium-independent since nicotinic stimulation can cause a reduction in cytoskeletal actin in the absence of extracellular calcium (Burgoyne *et al.*, 1989); as can antigenic stimulation in RBL-2H3 cells (Pfeiffer *et al.*, 1985); and raising intracellular calcium with ionomycin does not produce an increase in F-actin in RBL-2H3 cells (Apgar, 1991). PKC is implicated in cortical actin disassembly by the observations that metabolic inhibition of mast cells increases the calcium requirement for F-actin disassembly, while PKC activators cause a reduction in cytoskeletal F-actin in mast and chromaffin cells (Koffer *et al.*, 1990; Burgoyne *et al.*, 1989) and a rise in total F-actin in RBL-2H3 cells (Apgar, 1991). In addition to the phosphorylation of myosin light chain by MLCK, both the light and heavy chains of myosin are phosphorylated by PKC in RBL-2H3 cells within one minute of antigen stimulation (Peleg *et al.*, 1992). This could be a permissive step in cortical actin disassembly.

Scinderin and gelsolin, two calcium-dependent actin filament-severing proteins, both colocalise with actin to the cortical ring. Upon chromaffin cell stimulation scinderin, but not gelsolin, redistributes and colocalises with F-actin to patches around the cell periphery (Trifaró *et al.*, 1992). It has been proposed that calcium-activated scinderin creates zones in the subplasma membrane region where

actin filaments are severed and access to the plasma membrane is facilitated for secretory granules (Trifaró *et al.*, 1992).

Both fodrin and α-actinin, which anchor cytoskeletal elements to membranes, are present on the cytoplasmic surfaces of secretory granule and plasma membranes. Fodrin has been shown to redistribute into patches around the cell surface upon stimulation of chromaffin cells (Perrin and Aunis, 1985). Most secretory granules in chromaffin cells are located in the inner cytoplasm where they may interact with the cytoskeleton through fodrin and α-actinin. Some granules are attached to microtubules which may be involved in transporting them to the plasma membrane (Nakata and Hirokawa, 1992), although this is unlikely to be the case in mast cells, as taxol (a microtubule-stabiliser) has no effect on secretion (Dr. A. Koffer, personal communication). Cleavage of fodrin by the calcium-activated protease calpain I, would allow the rearrangement of F-actin of the sub-plasma membrane cytoskeleton and release of the secretory granules. However, no fodrin cleavage products are detected after maximal stimulation of secretion from chromaffin cells or parotid acinar cells (Perrin and Söling, 1992).

In addition to physically restraining secretory granules, the cytoskeleton may affect properties of the membrane that are relevant to its fusion with secretory granules: interaction with microfilaments is thought to influence some plasma membrane ion transporters in chromaffin cells (Lelkes *et al.*, 1986).

1.7.4 Phosphorylation and exocytosis

Phosphorylation and dephosphorylation provide well established mechanisms of control for many cellular processes. They are also believed to be important to secretion. While there is certainly an increase in phosphorylation of some proteins when cells are stimulated to secrete (for example chromaffin granule proteins, Creutz *et al.*, 1987), dephosphorylation may also be important since the phosphatase inhibitor okadaic acid strongly inhibits secretion from intact mast cells in response to IgE (Botana and MacGlashan, Jr., 1993) and from permeabilised mast cells in response to GTPγS in some circumstances (Churcher *et al.*, 1990b). Maximum inhibition of the IgE-

mediated response by okadaic acid is observed without any reduction in the rise in intracellular calcium, suggesting that a dephosphorylation event in the later stages of secretion (i.e. after signal transduction), perhaps of the exocytotic machinery, is necessary for IgE-mediated secretion. Perhaps cycling between a phosphorylated and a dephosphorylated state is necessary for continued exocytosis (the small amount of exocytosis that occurs in the presence of okadaic acid could result from phosphorylated components that then need to be dephosphorylated to recycle).

1.7.4.1 Tyrosine phosphorylation

Several groups have investigated tyrosine phosphorylation following cross-linking of the IgE receptor (FcεR1) on the surface of RBL-2H3 cells (Benhamou *et al.*, 1990, Li *et al.*, 1992, Deanin *et al.*, 1991). Several proteins, including phospholipase Cγ1, are phosphorylated on tyrosine within 1 minute of stimulation, some of these remain phosphorylated for up to 30 minutes, others are likely to be associated with initial signal transduction events. There is some evidence that an unidentified 40kd protein that is phosphorylated in response to a variety of secretagogues (antigen, calcium ionophore, thapsigargin, carbachol) is a member of the MAP kinase family (Santini and Beaven, 1993). Inhibitors of tyrosine phosphatases cause an increase in tyrosine phosphorylation of this 40kd protein and block secretion at a stage downstream of PKC activation and calcium mobilisation (Santini and Beaven, 1993). This suggests that both tyrosine phosphorylation and dephosphorylation are important regulatory steps. A tyrosine phosphatase has also been shown to enhance Ca^{2+}-dependent secretion from pancreatic acinar cells (Jena *et al.*, 1991).

1.7.4.2 Protein kinase C

RBL-2H3 cells contain five of the seven known PKC isoforms (α, β, δ, ε and ζ) which all become associated with the plasma membrane to varying degrees when RBL-2H3 cells are stimulated to secrete (Ozawa *et al.*, 1993a). This translocation, which is maintained for over 30 minutes, is dependent on extracellular calcium for isoforms α and β which have Ca^{2+}-binding domains, but not for the calcium-independent δ, ε and ζ

forms. It has been demonstrated that the β and δ isoforms will restore a calcium-dependent secretory response to permeabilised RBL-2H3 cells whose PKC has been lost by washing (Ozawa *et al.*, 1993a), while the α and ε isoforms appear to regulate PLCγ1 negatively (by reducing tyrosine phosphorylation of the enzyme) (Ozawa *et al.*, 1993b).

The role of PKC in exocytosis from this and other cell types is, however, controversial. Acute activation of PKC potentiates histamine release from RBL-2H3 and mast cells induced by the calcium ionophore A23187 (Beaven *et al.*, 1987; Katakami *et al.*, 1984) and significantly increases the calcium sensitivity of secretion from permeabilised chromaffin and mast cells (Knight *et al.*, 1988; Terbush and Holz, 1990; Howell *et al.*, 1989). Chakravarty *et al.*, (1990) found that activating protein kinase C with the phorbol ester TPA actually stimulated the release of up to 50% of the histamine content of rat peritoneal mast cells. However the release took place over 120 minutes, which is much slower than in response to conventional secretagogues.

Down-regulation of PKC by phorbol ester treatment for 24 hours substantially reduces secretion in response to Ca^{2+} from permeabilised chromaffin cells (Burgoyne *et al.*, 1988) and of nicotine-induced secretion from intact chromaffin cells (Wilson, 1990). Paradoxically the secretory response to nicotine recovers to normal levels in 4 days, although PKC activity does not, which suggests that loss of PKC is not responsible for inhibition of secretion. These results are further complicated by the finding that 24 hour exposure of chromaffin cells to TPA caused 90% loss of PKC from the cytosolic pool, but a concomitant 2-3 fold increase in membrane-bound PKC (Terbush and Holz, 1990). In RBL-2H3 cells exposure to PMA for 6 hours results in the degradation of only the calcium-dependent PKC isoforms (α and β) (Ozawa *et al.*, 1993a). This reduces but does not abolish exocytosis, which remains dependent on extracellular calcium (indicating a role for calcium other than the translocation of PKC).

If PKC activity is obligatory to the process of exocytosis then its inhibition should abolish exocytosis. Terbush and Holz (1990) found that inhibiting PKC (with staurosporine or a pseudo substrate peptide inhibitor), while removing the TPA-enhancement of Ca^{2+}-stimulated secretion, had no effect on release from chromaffin cells stimulated by 1μM Ca^{2+}. They conclude that PKC is not essential to exocytosis.

This work contradicts that of Knight *et al.* (1988) and Burgoyne *et al.* (1988) who found that a variety of non-specific PKC inhibitors inhibit not only the TPA enhancement but also the underlying Ca^{2+}-induced secretion from permeabilised chromaffin cells. Takei and Endo (1993) also found that a staurosporine-related compound strongly inhibited histamine release from rat mast cells induced by IgE, A23187 and TPA.

Since most protein kinase C inhibitors or down-regulators are notoriously non-specific, probably the most reliable results are those using the pseudo substrate peptide inhibitor which suggest that PKC is modulatory but not essential to exocytosis in chromaffin cells. The extent to which this is the case may well vary between cell types. A phosphorylation performed by PKC in the resting state before stimulation may be necessary to maintain the secretory apparatus in a secretion competent state. Thus activation of PKC would potentiate secretion and the inhibition of secretion reported by Burgoyne *et al.* (1988) who preincubated chromaffin cells for 2 hours with sphingosine, could be due to loss of this primed phosphorylated state. This is also consistent with the observations that: mast cells permeabilised more than 30 seconds before the addition of Ca^{2+} and GTPγS rapidly lose the ability to secrete; that this is completely restored by the addition of 100μM ATP; and that this effect is prevented by the DAG analogue AMG-C_{16} (a specific PKC inhibitor) (Churcher *et al.*, 1990b). The results are probably confused by the different effects of activators, down-regulators and inhibitors on the different PKC isoforms, some of which have inhibitory roles (preventing the activation of PLCγ1), some of which are stimulatory.

1.8 Annexin II, vesicle aggregation and exocytosis

What are the calcium-responsive proteins at the distal end of the secretory pathway? Annexin II is an attractive candidate. Annexin II has the lowest calcium requirement of the annexins studied for binding to all types of phospholipid vesicles (Blackwood and Ernst, 1990). The annexin II_2p11_2 tetramer is associated with chromaffin granule membranes as well as the plasma membrane *in vivo* (Drust and Creutz, 1991) and can aggregate chromaffin granules at physiological calcium concentrations (and fuse them in the presence of arachidonic acid) (Drust and Creutz,

1988). Located just under the plasma membrane it is ideally situated to help in the membrane fusion events of exocytosis. Electron-microscopy studies with purified annexin II and liposomes depicted cross-linking strands between liposomes (Nakata *et al.*, 1990). Similar structures could be seen linking chromaffin granules and the plasma membranes of chromaffin cells stimulated to secrete. Involvement in exocytosis is also implied by the observation that annexin II is phosphorylated within 5 minutes when chromaffin cells are stimulated to secrete (Creutz *et al.*, 1987). Annexin II has the highest binding affinity for phosphatidylinositol and phosphatidylserine which are found primarily in the inner leaflet of the plasma membrane. If self-association is the key to membrane aggregation by annexins, then annexin II has a head start. *In vivo* annexin II exists as a tetrameric complex consisting of a p11 dimer cross-linking two molecules of annexin II. The two annexin II molecules could each bind to a different vesicle membrane thus bringing the two vesicles into close apposition. If we assume that annexin II works in the same way as proposed for annexin VII (section 1.3.2), then once the first Ca^{2+}- binding sites are occupied, annexin II binds to secretory vesicles and can stimulate aggregation immediately without the need for further calcium rises to induce self-association. All of these observations make annexin II a good candidate for involvement in the exocytotic fusion of vesicles with the plasma membrane.

Annexin II in the monomeric or tetrameric form can almost fully reconstitute secretory capability in chromaffin cells that have been permeabilised for 25 minutes (Ali *et al.*, 1989b). This effect is greatest at 10μM Ca^{2+} while at sub-saturating Ca^{2+} (0.3-3μM Ca^{2+}) annexin II has no effect. Wu and Wagner (1991) achieved little effect using purified annexin II and found that annexin II-depleted cytosolic proteins were as capable of restoring Ca^{2+}-dependent secretory ability to permeabilised chromaffin cells as total cytosolic proteins. They were unable to reproduce the effects seen by Ali *et al.* (1989b) and consider that annexin II probably only has a modulatory role in exocytosis. However since cells retain approximately 50% of annexin II after 25 minutes permeabilisation, annexin II may not be limiting in the response to 1μM Ca^{2+} but becomes so in the response to 10μM Ca^{2+}. Other workers have found that annexin II needs to be phosphorylated, probably by PKC, to be active in this assay (Sarafian *et al.*,

1991). In addition, short peptides based on the C-terminus and the endonexin-fold regions of annexin II have been shown to cause partial inhibition of exocytosis from chromaffin cells (Ali *et al.*, 1989b; Roth *et al.*, 1993), although an N-terminal peptide had no effect (Ali and Burgoyne, 1990).

It is likely that many, proteins are necessary for exocytosis in response to a rise in intracellular calcium concentration, of which annexin II is only one. Certainly the ability to reconstitute Ca^{2+}-dependent exocytosis in run-down cells has been attributed to several other proteins including 14-3-3 proteins (Exo1) and the catalytic subunit of PKA (Exo2) (Morgan and Burgoyne, 1992; Morgan *et al.*, 1993) in chromaffin cells; p145 (Walent *et al.*, 1992) and PI-TP (Hay and Martin, 1993) in PC12 cells; ARF in HL-60 cells (Cockroft *et al.*, 1994); and gelsolin (Dr. A. Koffer, personal communication) and FOAD 1 and FOAD 2 (rac2 complexed to rho GDI) in mast cells (Dr. A. O'Sullivan, personal communication).

No protein receptor has been found for annexin II in the vesicle membrane, it simply binds to the phospholipid component. This begs the question of how the interaction can be specific to the correct membrane. Specificity is probably provided by the localisation of the tetramer through interaction with the cytoskeleton. However, monomeric annexin II remains cytosolic - how is this prevented from interacting with the nearest membrane? Annexin II's preference for phosphatidylserine and phosphatidylinositol, which are most abundant in the inner leaflet of the plasma membrane and the outer leaflet of granule membranes, may be important in this respect. Alternatively, the phosphorylation of annexin II observed in stimulated chromaffin cells (Creutz *et al.*, 1987) may be of monomeric annexin II to reduce its affinity for membranes.

1.8.1 Other annexins and exocytosis

Annexins I, II, IV, V, VI and VII can all bind to chromaffin granules at μM Ca^{2+} levels (Burgoyne, 1992) and therefore may do so in stimulated cells. However only annexins I, II and VII can aggregate vesicles. There is evidence that annexin I plays a part in vesicle trafficking: it is associated with chromaffin granule membranes and *in*

vitro can promote granule aggregation (Creutz *et al.*, 1987). Annexin I appears to be the factor in the cytosol of human neutrophils responsible for aggregation of neutrophil vesicles (Meers *et al.*, 1992, Meers *et al.*, 1993). Phosphorylation of serine residues in annexin I is detected in chromaffin cells soon after exposure to secretagogues, and persists until secretion terminates after 30 minutes (Michener *et al.*, 1986). Phosphorylation of the N-terminal tail of annexin I reduces its ability to aggregate chromaffin granules at sub-maximal Ca^{2+} concentrations (Wang and Creutz, 1992). It does not achieve this by inhibiting binding to granules, since the calcium requirement for binding to granules is slightly lowered. The phosphorylated form inhibits the unphosphorylated form's ability to aggregate granules, suggesting that, unless the two are simply competing for space to bind on the granule surface, that some form of loose dimerisation or oligomerisation occurs between annexin I molecules (on different granules) and that phosphorylation prevents this interaction. Therefore, unless annexin I cycles through a phosphorylated and a dephosphorylated state it is unlikely to be involved in membrane aggregation during exocytosis.

Annexin VII (synexin) is another popular candidate for playing a part in membrane fusion during exocytosis. It is present in adrenal chromaffin cells and has been localised to chromaffin granules (Kuijpers *et al.*, 1992). It can bind and aggregate chromaffin granules and rat pancreatic zymogen granules in a calcium-dependent manner *in vitro*, but the calcium requirement for aggregation is over 100μM (Creutz *et al.*, 1992b). Proponents of the role of annexin VII in membrane fusion point out that free cis-unsaturated fatty acids have a significant influence on the ability of annexin VII to fuse chromaffin granules: 1-2 % arachidonic acid is sufficient (Creutz, 1981), and since arachidonic acid is produced by most stimulated secretory cells it could enable annexin VII to function at lower calcium concentrations than suggested by *in vitro* studies performed in the absence of arachidonic acid. However, it has been demonstrated in mast, chromaffin and RBL-2H3 cells that exocytosis does not require arachidonic acid production (Churcher *et al.*, 1990a; Morgan and Burgoyne, 1990; Collado-Escobar *et al.*, 1990).

There is circumstantial evidence that annexin VI has an inhibitory role in secretion: annexin VI is localised to many hormone secreting cells and secretory epithelia but expression is lost from mammary gland ductal epithelia when lactation starts (Clark *et al.*, 1991), at precisely the time that annexin II becomes expressed on the apical plasma membranes of mammary epithelial cells (Handel *et al.*, 1991). In addition to this, annexin VI not only inhibits the calcium-induced fusion of PS vesicles (Blackwood and Ernst, 1990), but reduces the ability of annexin II and annexin VII to aggregate certain secretory granules (Creutz *et al.*, 1992b).

Factors such as the osmotic strength of the cytosol and the granule contents, phosphorylation of the annexins themselves and activation or appearance of cofactors could all influence the activities of annexins in ways we do not yet understand. Changes in membrane phospholipid composition influence the ability of membranes to fuse (Chernomordik *et al.*, 1993) and to bind annexins, so localised changes in lipid composition could influence membrane interaction with annexins. Without being able to define all of the internal conditions that occur in the cell, such as the effective local calcium and phospholipid concentrations an annexin is exposed to, it is difficult to rule out participation of any particular annexin in membrane trafficking events.

Although there is a body of evidence for a role for annexin II in exocytosis, it relies on work performed with isolated granules and permeabilised cells. The work in this thesis was designed to examine further at the possible participation of annexins in exocytosis from the secretory mast cell line RBL-2H3, and in particular to investigate the effect of loss of annexin II on intact cells, by specifically reducing expression of annexin II in the chosen cell line.

Chapter 2

Materials and methods

2.1 Cell lines

RBL-2H3 rat basophilic leukaemia cells were gifts from Dr. M. Hide of King's College London and Dr. D. Bar-Sagi of Cold Spring Harbour, U.S.A..

Jurkat (J6) human T cell leukaemia lymphoblasts were obtained from Dr. D. Cantrell at I.C.R.F., Lincoln's Inn Fields, London.

A431 human squamous carcinoma cells were obtained from Ms. M. Harrison at the I.C.R.F. cell production unit, Lincoln's Inn Fields, London.

Swiss 3T3 mouse embryo fibroblasts were obtained from Dr. E. Rozengurt, I.C.R.F..

PC12 rat adrenal pheochromocytoma cells were obtained from the European Collection of Animal Cell Cultures, Porton Down, Salisbury.

2.2 Cell culture

All cell types, except Jurkat, were routinely grown in 90mm dishes. RBL-2H3 cells were cultured in RPMI-1640 medium supplemented with 15% (v/v) foetal calf serum (FCS), penicillin (42U/ml), streptomycin (42µg/ml), Fungizone (2.2 µg/ml) and glutamine (1.7mM). Swiss 3T3 and A431 cells were grown in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% (for Swiss 3T3) or 5% (for A431s) FCS, penicillin (42 U/ml), streptomycin (42µg/ml), and glutamine (1.7mM). Jurkat cells were grown in flasks in RPMI-1640 medium supplemented with 5% FCS and penicillin, streptomycin and glutamine as above. PC12 cells were grown in DMEM supplemented with 10% horse serum, 5% FCS, Fungizone (2.2 µg/ml) and penicillin, streptomycin and glutamine as above.

A431s and Swiss 3T3 cells were maintained in a humidified atmosphere at 37°C in 10% CO_2. PC12 cells, Jurkat and RBL-2H3 cells were maintained in a humidified atmosphere at 37°C in 5% CO_2. All cells were regularly passaged and maintained at sub-

confluent levels. Cell culture reagents were obtained from Gibco BRL unless otherwise noted. Frozen stocks of all cell lines were kept in 90% FCS, 10% (v/v) dimethyl sulfoxide (DMSO) in liquid nitrogen.

PC12 cells were grown on Falcon dishes coated in polyornithine to improve cell attachment. To coat dishes, 2-3mls of sterile-filtered poly DL-ornithine (Sigma) (1mg/ml in water) was left on dishes overnight. Dishes were rinsed with sterile PBS before use. PC12 cells were induced to differentiate by supplementing the culture medium with 50ng/ml nerve growth factor (NGF) 2.5S from mouse submaxillary glands (Boehringer Mannheim).

2.3 Secretion experiments with permeabilised RBL-2H3 cells

RBL-2H3 cells were plated into 96-well plates on the day preceding the experiment, at an appropriate density to be 50-80% confluent by the following day. The growth medium was shaken off, and using a multichannel pipette each well was washed twice with 200µl PIPES-Cl buffer (50mM KCl, 50mM NaCl, 2mM $MgCl_2$, 20mM PIPES, 1mg/ml BSA, pH to 6.8 with NaOH). The cells were placed on ice and 50µl of ice-cold PIPES-Cl buffer containing 0.5 IU/ml of streptolysin-O (SL-O) (supplied in a reduced freeze-dried form by Murex) was immediately added to each well except for control wells. At 0°C SL-O binds to cell membranes but does not permeabilise them. After an 8 minute preincubation on ice to allow the SL-O to bind, the cells were washed twice with ice-cold PIPES-Cl buffer to remove contaminants in the SL-O solution. Still on ice, stimulation buffers were added to the appropriate wells to a final volume of 100µl.

Different calcium concentrations were achieved using Ca^{2+}-EGTA buffers at a final concentration of 3mM EGTA in PIPES-Cl buffer supplemented with 5mM ATP. Solutions of different GTPγS concentrations were made by diluting 100mM stock GTPγS (Gibco BRL) in PIPES-Cl buffer supplemented with 5mM ATP on the morning of the experiment. To obtain values for 100% secretion, 1% Triton X-100 (Sigma) was added to some wells. Cells were placed in a water bath at 37°C to trigger the permeabilising activity of SL-O and allow entry of secretagogues. Exocytosis was terminated after defined time periods by the addition of 100µl of 95.6 mM EDTA (determined to be of

equiosmotic potential with an osmometer). At the end of the experiment the 96-well plates of cells were spun at 5000g for 5 minutes to pellet any detached cells. 50µl of supernatant from each well was transferred to a clean black 96-well microtitre plate, to which 50µl of the flourogenic hexosaminidase substrate 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (Sigma), in citrate buffer at pH 4, with 0.01% Triton X-100 was added. The plate was then incubated at 37°C for 1-4 hours, depending on cell density. The reaction was terminated by the addition of 300µl of 0.2M Tris(hydroxymethyl)methylamine (Tris) (B.D.H.). Fluorescence was measured in a microplate fluorimeter, Titertek Fluoroskan II. The excitation wavelength was 355nm and emissions at 460nm were detected by a photomultiplier tube. The readings were analysed using spreadsheets in the SuperCalc 4.0 package (Computer Associates International Inc.).

As cellular components leak out of permeabilised cells their ability to secrete in response to secretagogues diminishes. This is known as 'run-down'. Run-down assays were performed on RBL-2H3 cells by triggering the permeabilising activity of SL-O (placing in a water bath at 37°C) for periods of between 0 and 30 minutes in 50µl of PIPES-Cl, before the addition of stimulation buffers.

2.4 Preparation of rat peritoneal mast cells

Mast cells were obtained in isotonic saline (0.9% NaCl, 1mg/ml BSA) by peritoneal lavage of 300g-plus adult male Sprague-Dawley rats. One rat was used per 96-well plate for secretion assays. Cells were pelleted at 5000g for 5 minutes and resuspended in 10mls of Pipes-Cl buffer (see above).

The cell suspension was filtered through nylon gauze to remove particulate matter such as hairs. and then gently layered over 1-2mls Percoll (Pharmacia). During centrifugation at 5000g the dense mast cells move to the bottom of the tube while contaminating blood cells remain on top of the Percoll. Most of the supernatant was removed by aspiration and cells were washed twice in 10mls of buffer.

To metabolically inhibit the cells they were resuspended in 1ml of PIPES-Cl buffer containing 10µl of 600mM 2-deoxyglucose (Sigma) and 1µl of antimycin A

(Sigma), and incubated at 37°C for 5 minutes. Cells were then mixed with 3ml of ice-cold SL-O solution (0.5 I.U./ml final) and kept on ice for 10 minutes. The cells were washed with buffer at 4°C, and resuspended in an appropriate volume of regeneration buffer (3mM creatine phosphate, 80μM ATP, 15μg/ml creatine kinase, pCa8). For run-down assays, rat mast cells were incubated at 37°C for periods of between 0 and 30 minutes before being triggered to secrete. 20μl of cells were aliquoted into each well of 96-well v-bottomed plates containing 60 μl of stimulation buffers of different concentrations of Ca^{2+} and GTPγS. The "100%" wells were lysed with 1% Triton X-100 (final). After 10 minutes, 120μl of PIPES-Cl buffer was added to each well, plates were spun at 5000g and 50μl of supernatant from each well was transferred to a clean black 96-well microtitre plate to which 50μl of the fluorogenic hexosaminidase substrate (see above) was added. Plates were incubated at 37°C for approximately 3 hours before the reaction was quenched with 300μl of 0.2M Tris.

Fluorescence was measured in a microplate fluorimeter, Titertek Fluoroskan II. The readings were analysed using the SuperCalc 4.0 package.

2.5 Cellular protein extraction

To prepare protein extracts, dishes of cells were washed in phosphate buffered saline (PBS) (10mM sodium phosphate pH 7.4, 147mM NaCl) (Gibco BRL) and placed on ice. Cells were then lysed with ice cold lysis buffer (10mM Tris-HCl pH 7.4, 150mM NaCl, 1% Nonidet P-40, 0.5mM PMSF and 1μM of the following small peptide inhibitors: chymostatin, leupeptin and pepstatin) for 2-3 minutes. Lysates were centrifuged at 10,000g for 5 minutes to pellet the nuclei. Supernatants were transferred to fresh tubes and stored at -20°C prior to use. For SDS-PAGE (section 2.7) aliquots were removed, mixed with 2 x sample buffer (0.125M Tris pH 6.8, 10% (v/v) glycerol, 4% (w/v) SDS, 0.001% bromophenol blue, 0.04g/ml DTT) and heated to 80°C for 10 minutes before loading.

To examine protein leakage from permeabilised cells, 3cm dishes of RBL-2H3 cells were treated as for secretion experiments, i.e. washed with PIPES-Cl buffer, incubated on ice with SL-O for 8 minutes, and washed again. The dishes were then

incubated at 37°C in 650µl PIPES-Cl buffer containing either 3mM calcium-EGTA buffer at pCa5 or 3mM EGTA (with 5mM ATP to mimic the conditions in secretion experiments). Samples (400µl) were removed from individual wells at various time points. Protein was precipitated from the samples by the addition of 550µl of ice-cold acetone and left on ice for 30 minutes. Insoluble material was pelleted in the minifuge for 5 minutes, most of the supernatant was removed with a fine tip and the rest was left to evaporate before resuspending the pellet in sample buffer. To determine protein remaining in the cells after permeabilisation the supernatant was removed by aspiration and the cells lysed directly into 2 x SDS-PAGE sample buffer.

2.6 Protein assay

Protein concentrations of samples in lysis buffer were determined using the Bio-Rad Protein Assay colour reagent. All samples were analysed in duplicate against bovine serum albumin (BSA) standards and a lysis buffer blank. Protein determinations of samples were used to ensure equal protein loadings on SDS-PAGE.

2.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). The gel running apparatus was made by Hoefer Scientific Instruments, Model SE 400. The acrylamide: bisacrylamide ratio was 30:0.8% (w/v) (Protogel, National Diagnostics) and the Protogel concentrations were 10% and 4% in the separating and stacking gels respectively. Gels were run at approximately 50 volts overnight or 150 volts during the day. SDS-PAGE was performed in the presence of the reducing agent 2-mercaptoethanol. Cellular protein extracts were run alongside pre-stained molecular weight markers purchased from Gibco BRL.

2.8 Western blotting

Western blotting was performed in an Electroblot Transfer Apparatus (Trans-blot™ Cell, Bio-Rad Ltd.) according to the manufacturer's instructions. Blotting onto

Immobilon-P (Millipore) was performed overnight at 0.25 A or during the day at 0.48 A, essentially as described by Towbin *et al.* (1979). The membrane was removed and 'blocked' in a 5% (w/v) solution of Marvel skimmed milk (Tesco) in PBS containing 0.05% (v/v) Tween-20 (PBS-Tween) for 1 hour. All subsequent steps were performed in PBS-Tween.

2.9 Immunoblotting

Blocked membranes were washed, placed in fresh PBS-Tween containing antibody raised against the protein of interest, and gently rocked for at least 4 hours, or overnight, at 4°C. Membranes were given three 5 minute washes in PBS-Tween and then incubated for 4-6 hours with a second antibody raised against the IgG species of the primary antibody. The different antibodies used and the antigens against which they were raised are as follows:

Primary antibodies

For *annexin I* — anti-p35I, immunoglobulin fraction of rabbit polyclonal antibody raised against human denatured placental annexin I

For *annexin II* — 1) αCal 1-15, raised in rabbit against a synthetic peptide of the first 15 residues of human annexin II, was a gift from Prof. Robert Burgoyne, Liverpool.

2) αp36I, the immunoglobulin fraction of rabbit polyclonal antibody raised against human denatured placental annexin II.

3) An affinity purified rabbit polyclonal antibody raised against porcine annexin II. This was a gift from Dr. Volker Gerke, Max Planck Institute, Goettingen.

4) HH7, a mouse monoclonal raised against a synthetic peptide of residues 1 to 18 of human annexin II. This was also a gift from Dr. V. Gerke.

5) mono-αp36, monoclonal ascites against native human placental annexin II. This was a gift from Dr. Clare Isacke, Imperial College, London.

For *annexin V* immunoglobulin fraction of rabbit polyclonal antibody raised against human placental annexin V

For *annexin VI* immunoglobulin fraction of rabbit polyclonal antibody raised against human placental annexin VI

All primary antibodies were used at a dilution of 1:500 apart from the anti-annexin I antibody, anti-p35I, which was used at 1:5000.

Secondary antibodies

Goat anti-rabbit IgG and goat anti-mouse IgG antibody conjugated to alkaline phosphatase were obtained from Sigma. Secondary antibodies were used at a dilution of 1:5000. Colour was developed with Western Blue (Promega).

2.10 Immunofluorescent labelling of annexin II *in situ*

RBL-2H3 cells were fluorescently labelled for annexin II according to the method of Thiel *et al.* (1992). Cells were grown on sterile glass coverslips in the usual conditions. When 25-50% confluency was reached, medium was removed by aspiration and the cells were fixed in 3.7% formaldehyde in PBS at room temperature for 15 minutes. Cells were then treated with methanol for 6 minutes at -20°C before permeabilisation in ice-cold cytoskeletal stabilising buffer, consisting of 0.5% Triton X-100, 100mM KCl, 3mM $MgCl_2$, 1mM $CaCl_2$, 200mM sucrose, 10mM Hepes, pH 6.8, 1mM PMSF, for 2 minutes; followed by two 5 minute washes in the same buffer without Triton X-100. Subsequently, the cells were placed in PBS at room temperature for 4 minutes and then methanol for 6 minutes at -10°C. The coverslips were then rinsed in PBS and placed in an airtight box on a piece of damp Whatman 3MM paper. A series of dilutions from 1 in 25 to 1 in 250 of the primary antibody (mouse monoclonal anti-annexin II, HH7) in PBS were used to determine the optimum concentration. 50µl of each dilution was put on a different coverslip and incubated at 37°C for 45-60 minutes. Coverslips were washed in PBS for 3 minutes before covering with secondary antibody for 45 minutes at 37°C. The secondary antibody was either FITC-conjugated anti-mouse IgG (Sigma) at 1 in 200 for direct visualisation, or biotinylated anti-mouse IgG (Vectastain) at 1 in 200. For the latter a third incubation followed, of 45 minutes at 37°C

with a streptavidin-Texas Red conjugate (Calbiochem) at 1 in 300. After a final wash in PBS, cells were mounted on glass slides in glycerol containing 2% propyl gallate. The fluorescence labelling was observed using a Leitz fluorescence microscope. Photographs were taken on AGFApan 400ASA 35mm film.

2.11 Bacteriophage lambda cDNA library screening

Annexin II has been cloned from several species including human, cow, mouse and *Xenopus laevis*. For antisense transfection experiments in a rat cell type it was necessary to clone rat annexin II.

A bacteriophage lambda library (a gift from John Adelman, Oregon Health Sciences University) derived from RBL-2H3 cells was screened for annexin II. The lambda library (80,000 plaques) was plated out using Y1090 as host bacteria. After incubation overnight at 37°C duplicate lifts were made on Hybond-N membranes (Amersham). DNA was crosslinked to the membranes by exposure to short-wave U.V. light (302nm) for two minutes. The filters were probed using a murine annexin II cDNA (a gift from Tony Hunter, Salk Institute) labelled with [^{32}P]-αdCTP (3000 Ci/mmol, Dupont) using the USB Random Primed DNA Labelling Kit. Membranes were prehybridised in a rotating oven at 63°C for 30 minutes in 0.5M sodium phosphate buffer pH 7.2 containing 7% SDS, 1mM EDTA, before addition of the probe. Labelled probes were ethanol precipitated, boiled for three minutes and then cooled on ice before addition to the hybridisation solution. After overnight hybridisation, membranes were given one 10-minute wash in 0.04M sodium phosphate buffer pH 7.2 containing 5% SDS and three 20-minute washes in 0.04M sodium phosphate buffer pH 7.2 containing 1% SDS. Membranes were washed at low stringency (50°C) to facilitate cross-species hybridisation. Dried membranes were exposed to Kodak X-Omat AR X-ray film with double intensifying screens, at -70°C. Unfortunately, and despite several rounds of screening, no positive clones were identified in this library.

2.12 Plasmid cDNA library screening

Due to the lack of success with the bacteriophage library a second library was

screened. The RBL-2H3 plasmid cDNA library, a gift from Dr. Ernie Peralta, Harvard, was constructed in the plasmid pcDNA I (Invitrogen). The plasmid library was plated out (30,000 colonies per plate) on Hybond-N on bactoagar. After incubation overnight at 37°C two replicates were made from the master and cultured for a further 8 hours. The two daughter filters were placed in 10% (w/v) SDS to lyse the bacteria *in situ*. The membranes were fixed and washed according to Sambrook *et al.* (1982) and DNA was crosslinked to the membranes by exposure to U.V. light (302nm) for two minutes. Membranes were probed with murine annexin II cDNA as described above. Bacterial colonies which gave positive signals on both films were subjected to a second round of screening. Twelve positive colonies were identified by aligning the master with spots on the autoradiographs. Each colony was scraped into 1ml of Luria broth (LB) and incubated at 37°C for 1 hour. Using $O.D._{600}$ measurements (1 $O.D._{600}$ unit is equivalent to 8×10^8 cells/ml) the bacteria were diluted before plating on to Hybond-N laid over LB-agar to ensure adequate separation of colonies. Plates were incubated overnight and screened as detailed above. Six plates yielded positive colonies, one from each was chosen for further analysis.

2.13 Large and small scale plasmid DNA preparations

Terrific Broth (TB), LB, agar and other reagents for bacterial culture were purchased from Gibco BRL. Large and small scale plasmid DNA preparations were obtained by alkaline lysis according to Sambrook *et al.* (1982). Large scale preparations were purified either by precipitation with polyethylene glycol (PEG), or by equilibrium centrifugation in a CsCl-ethidium bromide gradient as detailed in Sambrook *et al.* (1982). Nucleic acid solutions were quantified by taking $O.D._{260}$ readings in a Pharmacia Ultrospec II spectrophotometer (1 $O.D._{260}$ unit is equivalent to 50µg/ml DNA).

2.14 Construction of plasmids for transfection and for sequencing

(i) *Restriction enzyme digests*

Restriction enzymes were obtained from either ProMega, Gibco BRL or

Boehringer Mannheim, and were used according to the manufacturers' instructions, in appropriate buffers.

(ii) *Purification of DNA fragments from agarose gels*

Digests were resolved on 1% agarose gels in 40mM Tris-acetate pH 7.5, 1mM EDTA pH 8 (TAE) electrophoresis buffer. After visualisation with ethidium bromide, the required fragment was excised from the gel with a clean scalpel blade and recovered from the gel slice using Geneclean^R II (Bio 101, La Jolla, Ca.). Exposure times to U.V. were kept to a minimum to avoid damaging the DNA.

(iii) *Ligation of DNA fragments*

For blunt-end ligations, 3' recessed termini were converted to blunt ends by T4 DNA polymerase (ProMega), and 5' recessed termini were converted to blunt ends by Klenow DNA polymerase (ProMega). Vectors were dephosphorylated with calf intestinal phosphatase (CIP) (Sigma) according to the manufacturer's instructions. Fragments were incubated at 4°C (blunt end) or 15°C (sticky end) overnight with T4 DNA ligase (ProMega, Gibco BRL). For blunt end ligations, PEG was included in the ligase buffer to facilitate the reaction, and insert was present in 5-fold molar excess over vector. Sticky-end ligations were performed with a 3-fold molar excess of insert. Generally a ligation mixture containing vector alone was used as a control to assess the degree of self-ligation.

2.15 Preparation and transformation of competent *E.coli*

Competent bacteria were prepared by two different protocols:

(i) *$CaCl_2$ method (for use with sticky-end ligations)*

Fresh competent bacteria were prepared by growing a 40ml culture of *E.coli* strain XL1-Blue (Stratagene) in 1 x TB until the O.D.$_{600}$ was approximately 0.3. The bacteria were centrifuged at 6000g for 5 minutes at 4°C, resuspended in 20ml of ice-cold 50mM $CaCl_2$ and left on ice for 20 minutes. Cells were pelleted again and resuspended in 4ml of ice-cold 50mM $CaCl_2$. The bacteria were then left for several hours (preferably overnight) at 4°C,, before addition of DNA. Aliquots (0.3ml) were mixed with 5µl of ligation reaction and kept on ice for 4-6 hours. These were heat-shocked by incubating

at 42°C for 90 seconds and returned to ice before gently pelleting and pouring off the $CaCl_2$. The bacteria were resuspended in 600µl of TB and left at 37°C for 45 minutes before being plated out on agar containing an appropriate antibiotic for selection of transformed bacteria, and incubated at 37°C overnight.

(ii) *High Voltage Electroporation (for use with blunt-end ligations)*

For blunt-end ligations (which occur with lower efficiency) transformation by high-voltage electroporation was employed. Electrocompetent bacteria were prepared by adding a 10ml overnight culture of XL1-Blue to 200ml of TB and incubating with vigourous shaking at 37°C until the $O.D._{600}$ of the culture reached 0.65. Bacteria were harvested at 6000g for 5 minutes at 4°C and gently washed with sterile ice-cold 10% (v/v) glycerol 4-5 times. The cells were finally resuspended in 3ml of 10% glycerol and stored at -70°C in small aliquots. For transformation, frozen cells were thawed on ice immediately before use. A 60µl aliquot of cells was mixed with 1.5µl of ligation reaction and electroporated by a single pulse from a 50µF capacitor charged to 1500V in an ElectroPorator (Invitrogen Corp.). Cuvettes (2mm) for use with bacterial cells were supplied by Bio-Rad. Immediately after electroporation, 1ml of pre-warmed SOC medium (Sambrook *et al.*, 1982) was added to the electroporated sample which was then left at 37°C for 45 minutes. 200µl was removed and plated out on LB-agar containing the appropriate antibiotics, and incubated at 37°C overnight.

For constructs in pBluescript (Stratagene), blue/white colour selection was used to detect plasmids containing inserts. To induce expression of β-galactosidase, agar plates were spread with 12µl of 200mg/ml of isopropyl-beta-thio-galactopyranoside (Sigma) and 50µl of 20mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Sigma).

Small scale preparations of individual colonies were analysed by restriction enzyme mapping to determine the presence and orientation of inserts.

2.16 Nucleic acid sequencing

DNA sequencing was performed according to the dideoxy-chain termination method of Sanger *et al.* (1977) using the Sequenase^R Version 2.0 kit (USB) with [^{35}S]-

αdATP (1000 Ci/mmol, Dupont). Polyacrylamide gel electrophoresis was performed using the National Diagnostics' SequaGel™ Sequencing System. Gels were usually 6% acrylamide and 8.3M urea, but to read up to 450-500 bases from the primer 4% gels were used. Gels were run for 2-5 hours at constant power, 40 W (gel temperature approximately 55°C), fixed for 20 minutes in 5% (v/v) acetic acid, 15% (v/v) methanol to remove the urea, and dried at 80°C. Sequencing gels were exposed overnight at room temperature to RX Fuji X-ray medical film.

2.17 Total cellular RNA extraction

Total cellular RNA was prepared from cells by the single step method of Chomcyzymski and Saachi (1987), using guanidinium thiocyanate-phenol-chloroform. Dishes of adherent cells were rinsed in sterile PBS before 1ml per 90mm dish of guanidinium thiocyanate solution was pipetted directly on to them. The cells were detached with a scraper and the solution transferred to an Eppendorf tube for phenol-chloroform extraction. Cells grown in suspension were centrifuged and washed, and guanidinium thiocyanate solution was added to the pellet. RNA yields were quantified by measuring $O.D._{260}$ (1 $O.D._{260}$ unit is equivalent to 40μg/ml of RNA).

2.18 RNA gel electrophoresis and northern blotting

RNA samples were diluted on ice with H_2O, and denatured by the addition of an equal volume of loading mix (50% formamide, 11% formaldehyde, 0.01M phosphate buffer). (0.5M Phosphate buffer (PB): 85g of Na_2HPO_4, 47.7g of NaH_2PO_4 per litre.) Samples were then heated to 60°C, quenched on ice and mixed with a 1/4 volume of PB-FDE (0.1M EDTA, 0.3g/ml Ficol, 0.5mg/ml bromophenol blue, 0.01M PB) before loading. Samples were run on 1% (w/v) agarose gels containing 6.4% (v/v) formaldehyde and 10mM PB. Gels were run for 3-12 hours at 25-70 V in 0.01M PB. For long running times the buffer was replaced every four hours.

RNA was transferred from agarose gel to Hybond-N membrane by capillary blotting. Two sheets of 3MM paper dipped in 20 x SSC (3M NaCl, 0.3M sodium citrate pH 7) were placed on a platform, with both ends acting as wicks in 20 x SSC in the tray

below. The gel was placed on top of this. A sheet of Hybond-N was placed on top of the gel followed by 4 sheets of 3MM paper dipped in 2 x SSC, and finally a stack of paper towels. This was generally left overnight with a small weight on top. The Hybond-N was placed on 3MM paper to dry before exposure to U.V. light (302nm) for 1 minute to cross-link the RNA to the membrane.

2.19 Labelling of DNA probes for northern blotting

cDNAs were excised from plasmids containing them with the appropriate restriction enzymes and isolated as described in section 2.14 (ii). They were labelled using the Random Primed DNA Labelling kit from U.S.B., according to the manufacturer's instructions. Approximately 25ng of each cDNA was labelled with 50μCi of [^{32}P]-αdCTP. The different cDNAs used were as follows:

annexin I rat annexin I cDNA from Dr. Yasuaki Shimizu, Yamanouchi Pharmaceutical Co., Ltd.

annexin II murine annexin II cDNA from Dr. Tony Hunter, Salk Institute, California

annexin III human annexin III cDNA from Dr. Jeff Browning, Biogen, Boston.

annexin IV bovine annexin IV cDNA from Dr. Carl Creutz, Department of Pharmacology, University of Virginia

annexin V human annexin V cDNA from Dr. Rudolf Hauptmann, Ernst Boehringer Institut fur Arzneimittelforschung, Wien

annexin VII human annexin VII cDNA from Dr. Carl Creutz, Department of Pharmacology, University of Virginia

annexin VIII human annexin VIII cDNA from Dr. R Hauptmann, Ernst Boehringer Institut fur Arzneimittelforschung, Wien

Overlapping annealed oligos of the human annexin VI cDNA supplied by Dr. Paul Smith, UCL were used to make a probe for annexin VI.

Northern blots were probed as described in section 2.11.

2.20 Poly A^+ RNA preparation

Poly A^+ RNA was extracted from total RNA preparations using Dynabeads Oligo $(dT)_{25}$ according to the manufacturer's instructions (Dynal UK Ltd.). Total RNA samples were mixed with a solution of magnetic beads coupled to poly-dT. The beads bind specifically to polyadenylated mRNA allowing them to be retained while other RNA species are removed by washing. mRNA is then eluted off the beads by altering the ionic conditions.

2.21 Primer extension analysis

To examine expression of isoforms of annexin II, primer extension analyses were performed using poly A^+ RNA isolated from several different cell lines. The amounts of poly A^+ RNA obtained were too small to quantify by O.D. readings so instead an aliquot of each poly A^+ RNA sample to be used was Northern blotted and probed with labelled rat annexin II cDNA. If a signal could be detected in this manner a similar quantity was used for primer extension. Two different oligonucleotide primers complementary to regions of rat annexin II cDNA, described in detail in Chapter 4.4.1, were obtained from the Molecular Medicine Unit, Kings College School of Medicine and Dentistry. Primer extension analysis was performed using Promega's Primer Extension System kit according to the manufacturer's instructions. Primers were end-labelled with an equimolar quantity of [^{32}P]-γATP by T4 polynucleotide kinase. To determine percentage incorporation of radioactivity into the primers 3μl of a 1:100 dilution of the labelling reaction was spotted onto each of two Whatman DE81 filters. Both were dried before one was given two 5 minute washes in 50ml of 0.5M Na_2HPO_4, pH 6.8 to remove unincorporated nucleotides. The amount of radioactivity on each filter was measured by Cerenkov counting in the ^{3}H channel of a liquid scintillation counter. The percentage incorporation was calculated as follows:

$$\frac{\text{cpm in washed filter}}{\text{cpm in unwashed filter}} \times 100 = \text{percentage incorporation}$$

The value varied between batches from 10-80%, but the probe was only used when the value was greater than 25%. Several attempts were made to determine the

optimum temperature and duration for annealing the end-labelled primer to mRNA (2 hours at 58°C). Reverse transcriptase transcribes from the primer to the 5' end of the mRNA and the primer extension products were analysed on a 6% polyacrylamide gel alongside a set of known sequencing reactions to allow exact sizing of the bands followed by autoradiography.

For later attempts the protocol was adapted to try and increase the signal. Instead of end-labelling the primer, radiolabelled dCTP was included in the reaction and the amount of unlabelled dCTP was reduced. The concentrations of nucleotides used were as follows: 1mM dATP, dGTP and dTTP; 5μM dCTP and 1μl of [^{32}P]-αdCTP (3000 Ci/mmol, Dupont) (approximately 1μM), other components of the reverse transcriptase reaction mixture were unchanged.

2.22 Vectors for transfection

(i) *pNV*

The expression vector pNV was a gift from Dafna Bar-Sagi, Cold Spring Harbour (figure 2.1). It contains the 5' and 3' long terminal repeats (LTR) from the Maloney Leukaemia Virus; these act as transcription promoter and polyadenylation signals for a neomycin resistance gene (for selection of successfully transfected clones). There is a single cloning site, Xho I, for the insertion of DNA. An ampicillin resistance gene (for beta-lactamase) and origin of replication are present for manipulations in bacteria. Plasmids for transfection were constructed by digestion to completion of pNV with Xho1, followed by blunt-ending and ligation to annexin I or II blunt-ended cDNA fragments as described in section 2.14. The orientation of inserts was determined by restriction mapping.

(ii) *pRc/CMV*

In later experiments transfection was performed using the vector pRc/CMV (Invitrogen) (figure 2.2). This plasmid has five unique restriction enzyme sites at its cloning site: Hind III, Bst XI, Not I, Xba I and Apa I. It has two promoters, one derived from cytomegalovirus and the other from SV40 virus early promoter. These drive transcription of the inserted DNA and the neomycin resistance gene respectively. There

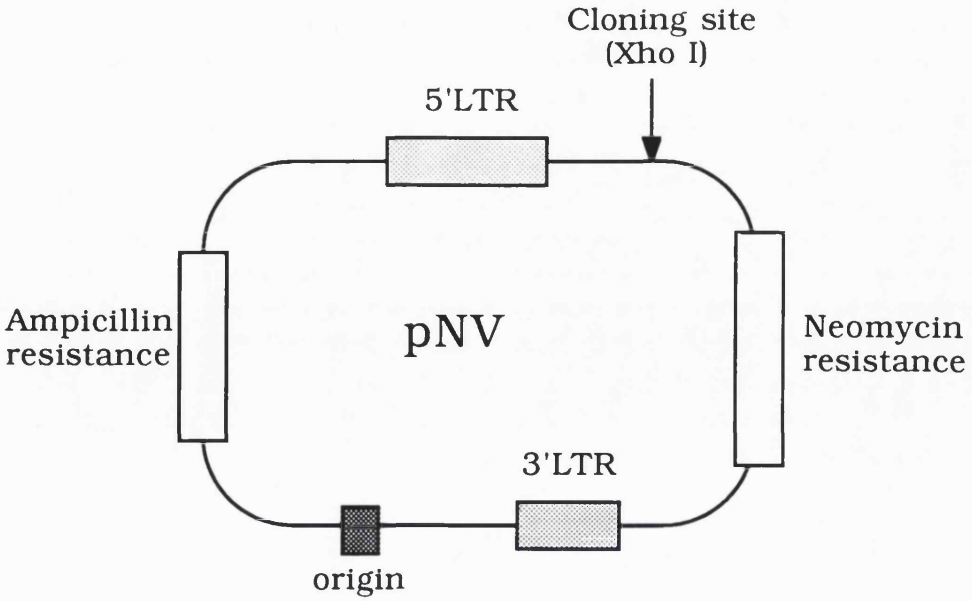


Figure 2.1 *The expression vector pNV.*
This schematic representation of pNV shows the approximate positions of the antibiotic resistance genes, the bacterial origin of replication, the cloning site and the viral sequences that act as promoter and polyadenylation signals for transcription in mammalian cells.

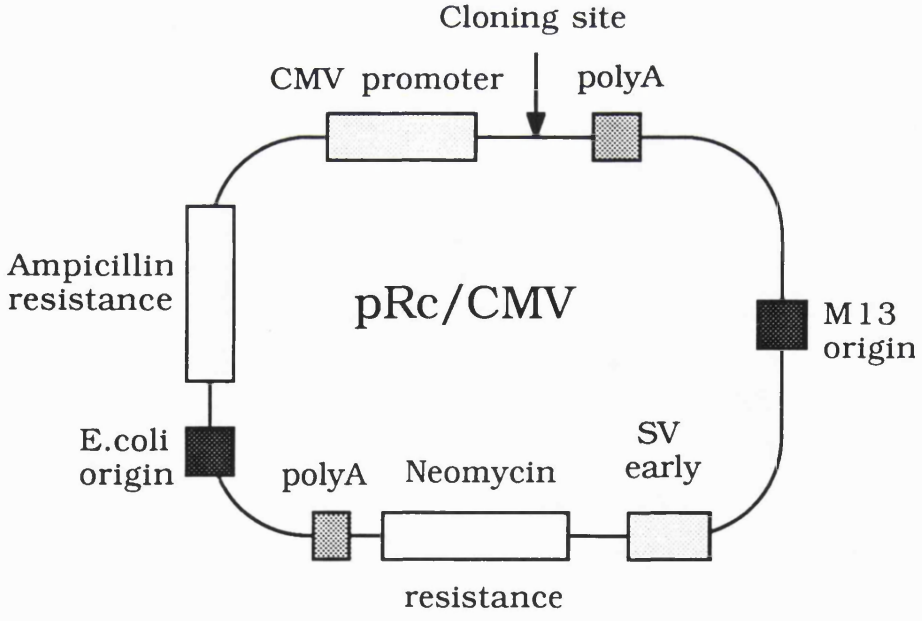


Figure 2.2 *The expression vector pRc/CMV.*
This schematic representation of pRc/CMV shows the approximate positions of the antibiotic resistance genes, the bacterial origin of replication, the M13 phage origin of replication,the cloning site and the two sets of viral sequences that act as promoters and polyadenylation signals for transcription in mammalian cells.

are also separate polyadenylation signals for each. The vector contains origins of replication and a beta-lactamase gene for propagation and selection in either M13 or E.coli.

Fragments of annexin I or II cDNA were excised from their host plasmids using restriction enzymes designed to allow directional cloning and sticky-end ligations where possible, otherwise the vector and fragments were blunt-ended and ligated as described in section 2.14.

2.23 Transfection of RBL-2H3 cells

To examine the effect of alterations in annexin II expression RBL-2H3 cells were transfected with plasmids containing sense or antisense annexin II cDNA. The antibiotic Geneticin^R or G-418 Sulphate (Gibco BRL), commonly known as neomycin, was used to select for stably transfected cells. Geneticin^R is an aminoglycoside that is toxic to eukaryotic and prokaryotic cells. The resistance gene, neomycin phospho-transferase, is bacterial in origin but is also effective when expressed in eukaryotic cells. Several different techniques for transfection were employed.

(i) *DEAE-dextran*

DEAE-dextran transfection of cells is generally recommended only for transient transfections, however it has been successfully used to create stable transfectants with RBL-2H3 cells (Dr. D. Bar-Sagi, personal communication). The ProFection™ Mammalian Transfection Systems kit (ProMega) was used. Each 90mm dish of approximately 50% confluent RBL-2H3 cells was washed with 25ml of serum free Eagles' Minimum Essential Medium (EMEM). This was aspirated from the dish before 3ml of EMEM containing 20μg/ml DEAE-dextran and 6μg of plasmid DNA for transfection was pipetted onto the cells. Dishes were returned to the culture incubator for 4 hours. Medium was then removed and the cells were shocked for 2 minutes at room temperature in 25ml HEPES-buffered saline (HBS) (137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 6mM glucose, 21mM HEPES pH 7.2) containing 10% DMSO. Dishes were then rinsed twice with 5ml of serum-free EMEM and replaced in the incubator in 10ml of fresh medium containing 15% FCS. The cells were incubated for 48 hours (to allow

expression of the transfected cDNAs) before stably transfected cells were selected by supplementing the normal growth medium with 350μg/ml Geneticin^R.

(ii) *$Ca_3(PO_4)_2$*

Transfection was also attempted in RBL-2H3 and Swiss 3T3 cells using the standard protocol for calcium phosphate-mediated transfection of adherent cells described in Sambrook *et al.* (1982). For each 90mm dish of approximately 25% confluent cells a calcium phosphate-DNA precipitate was prepared as follows: to 10μg plasmid DNA in 220μl of 0.1 x Tris-EDTA (1mM Tris-Cl, pH 8.0, 0.1 mM EDTA) was added 31μl of 2.0M $CaCl_2$, one drop at a time. This mixture was then added to 250μl of 2 x HBS, with gentle mixing. After standing at room temperature for 30 minutes a fine precipitate forms. This was gently resuspended by pipetting before adding the suspension to the medium on the cells, whilst carefully rocking the dish. The cells were returned to the cell culture incubator for 24 hours. The medium containing the calcium phosphate-DNA precipitate was then removed, the cells were washed once with PBS and given fresh medium. After a further 24 hours stably transfected cells were selected for by supplementing the medium with 350μg/ml Geneticin^R.

(iii) *Electroporation*

Confluent RBL-2H3 cells were harvested by trypsinisation, centrifuged, washed in PBS and resuspended in PBS at approximately 5 x 10^7 cells/ml. 5μg of plasmid DNA for transfection was added to 400μl aliquots of cells in sterile electroporation cuvettes and allowed to stand at room temperature for 10 minutes. Electroporation cuvettes (4mm) used with mammalian cells were supplied by British Technology Products Ltd. Power supply limits were set to 50mA and 50W. Cells were electroporated by a single pulse from a 500μF capacitor charged to 250V in an ElectroPorator (Invitrogen Corp.). After 1 minute cells were divided between two 90mm dishes each containing 10mls of culture medium and returned to the cell culture incubator. Stably transfected cells were selected for by the addition of 350μg/ml Geneticin^R to the culture medium after 48 hour recovery.

The culture medium was changed every two days as Geneticin^R breaks down slowly in solution, and to remove dead cell debris. Cell death was rapid and after about

8 days, individual colonies were visible to the naked eye. Individual clones were isolated using stainless steel cloning rings to trypsinise single colonies, or simply scraped off the dish with a sterile pipette tip. Cloned cell lines were maintained in medium containing 175µg/ml Geneticin^R.

2.24 Synthesis of strand-specific radiolabelled RNA probes

To examine the expression of antisense RNA in transfected RBL-2H3 cells, radiolabelled sense RNA was transcribed from rat annexin II cDNA fragments in pBluescript. Plasmids were first linearised to ensure that only the portion containing the cDNA was transcribed. Using the Promega In Vitro Transcription kit, RNA was transcribed from one of the RNA polymerase promoters (T3 or T7) on either side of the cDNA insert in pBluescript in a reaction mix containing 50µCi of [^{32}P]-αCTP at 10mCi/ml. Template DNA was removed by digestion with RNAse free DNAse (RQ1) for 15 minutes at 37°C, and unincorporated nucleotides were removed by precipitating the RNA with ammonium acetate.

2.25 Large scale synthesis of strand-specific RNA

To use as a positive control on Northern blots of transfected cells probed for the presence of antisense transcripts, antisense RNA was transcribed from the same template as above, using the promoter at the other end of the cDNA insert and cold ribonucleotides.

To determine the quantity of positive control RNA synthesised, 10µl of the 100µl large-scale synthesis reaction was transferred to a separate tube, as soon as all the components had been added, and mixed with 0.5µl of [^{32}P]-αCTP. Reactions were incubated for two hours at 37°C. 3µl of the radioactive reaction mixture was spotted onto each of two Whatman GF/C glass fibre filters and left to dry at room temperature (Sambrook *et al.*, 1982). Once dry, one filter was given three, two-minute washes in 200ml ice-cold 5% trichloroacetic acid, 20mM Na pyrophosphate. This filter was then dipped in 70% ethanol and left to dry at room termperature. The two filters were placed in separate scintillation vials and the amount of radioactivity on each filter was

measured by Cerenkov counting in the ^{3}H channel of a liquid scintillation counter. The percentage incorporation was calculated using the formula:

$$\frac{\text{cpm in washed filter}}{\text{cpm in unwashed filter}} \times 100 = \text{percentage incorporation}$$

The large scale synthesis reaction starts with 19µl of 2.5mM CTP, which is equivalent to 47.5 nmoles of CTP. The product RNA is approximately 900 bases in length. If we assume that one quarter of the bases in the product RNA are CTP then there will be 225 CTP molecules per product RNA molecule, or 225nmol CTP/nmol product RNA. Therefore if all of the starting 47.5 nmoles of CTP are incorporated into RNA there will be 47.5/225 = 0.209nmoles of product RNA.

Standard tables state that 1pmole of 1kb RNA wieghs on average 0.34µg, therefore 0.209 nmole of 0.9kb RNA weighs 209 x 0.9 x 0.34 = 63.95µg, which is the total possible amount of RNA synthesised. Multiplying this figure by the percentage incorporation determined above, gives the actual amount (in µg) of product RNA synthesised. Various loadings of positive control RNA were subjected to denaturing RNA gel electrophoresis alongside 20µg of RNA from each clone being screened. Gels were Northern blotted onto Hybond N and hybridised overnight at 64°C with radiolabelled sense strand RNA. The blots were washed as described in section 2.11 but at high stringency (67°C) as RNA hybrids are particularly stable.

2.26 Attenuation of annexin II expression using antisense oligonucleotides

A phosphothiorate 19 base-oligonucleotide complementary to a region covering the translation start site of the rat cDNA was purchased from the Molecular Medicine Unit, Kings College School of Medicine and Dentistry. Four identical 30mm dishes (A-D) of RBL-2H3 cells were plated out at approximatley 10% confluence. On day 1, 1ml of medium containing the oligonucleotide at a concentration of 50µg/ml (8µM) was placed on dish A, fresh medium was added to the other dishes. On day 2, medium containing the oligonucleotide was placed on dishes A and B, fresh medium was given to the other dishes. On day 3, fresh medium containing the oligonucleotide was placed on dishes A,

B and C; fresh medium was given to the control dish. On day 4 all dishes were trypsinised and lysed into 250µl of lysis buffer. Samples were subjected to SDS-PAGE electrophoresis, Western blotted and immunoblotted for annexin II.

Chapter 3

Characterisation of annexin expression and RBL-2H3 cell secretion

3.1 Annexin expression in mammalian cell lines

(i) *Northern blotting*

Several members of the annexin family have been implicated in secretion - annexins I, II, and VII (Meers *et al.*, 1993; Ali *et al.*, 1989b; Kuijpers *et al.*, 1992), and annexin VI in a negative regulatory capacity (Clark *et al.*, 1991). To establish which members of the annexin family are expressed in the secretory cell line chosen for this study, RBL-2H3 cells, RNA was prepared from RBL-2H3s and several other cell lines currently in use in our laboratory for comparison.

20μg of total RNA extracted from RBL-2H3, PC12 cells before and after 1-day and 3-day exposure to NGF, A431 and quiescent Swiss 3T3 cells were subjected to denaturing RNA gel electrophoresis, Northern blotted and probed for the presence of mRNA for annexins I to VIII. The cDNAs of annexins I to V, VII and VIII were excised from plasmids containing them with the appropriate restriction enzymes, extracted from agarose gels and labelled using a random primer oligo labelling kit. Blots were washed at low stringency to facilitate cross-species hybridisation. Autoradiographs of each blot are shown in figure 3.1. A satisfactory result was not obtained with the probe synthesised using annexin VI overlapping oligos so expression of this annexin was examined at the protein level by Western blotting (see section 3.1 (ii)). The expression of annexin mRNAs in the cell types examined can be qualitatively compared, but quantitative comparisons are only possible between those cell lines derived from the same species since the efficiency of cross-species hybridisation is not known and will vary between cDNAs.

Annexin I mRNA was detected in A431 and RBL-2H3 cells, and at very low levels in Swiss 3T3 cells. Annexin I was not detected in PC12 cells. Annexin II is one of the

most widely expressed members of the family in this study. It was detected in A431, RBL-2H3, Swiss 3T3 and PC12 cells induced to differentiate by NGF. It has previously been shown that annexin II is only expressed in Swiss 3T3 cells after serum stimulation (Keutzer and Hirschhorn, 1990). The band detected in the Swiss 3T3 track is extremely weak and since the probe was derived from cDNA of the same species this indicates that levels are extremely low. Those cell lines that were positive for annexin II mRNA also contained a second, larger transcript of unknown origin. If this is not an artefact it could represent a stable processing intermediate or possibly an alternatively spliced form.

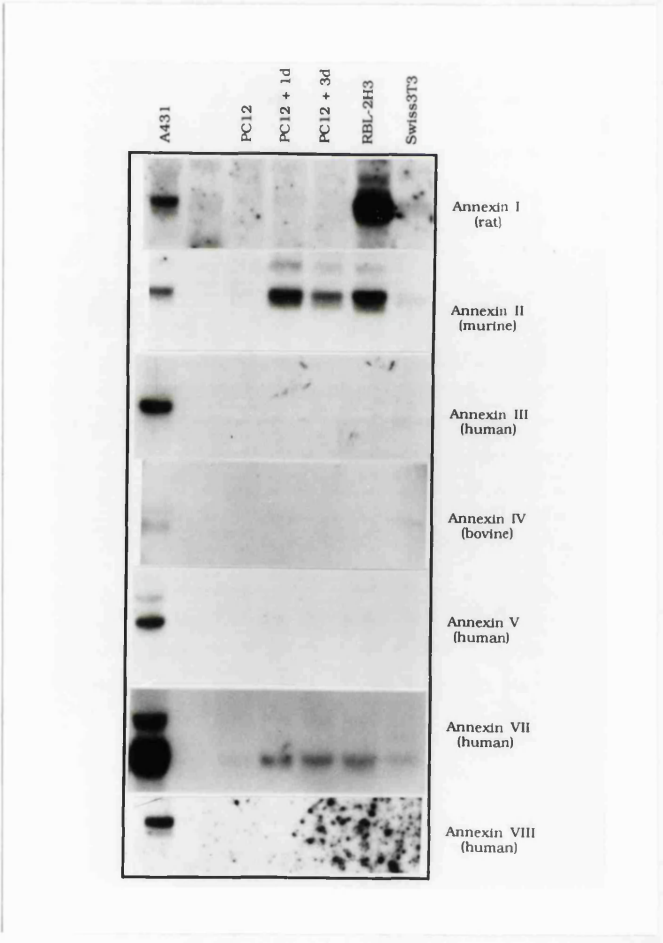


Figure 3.1 *Northern blots of RNA extracted from A431, PC12 (before and after differentiation induced by exposure to NGF for 1 or 3 days), RBL-2H3 and Swiss 3T3 cells, probed for annexins I- V, VII and VIII.*
Gels were loaded with 20µg of RNA per track. The second track from the left on each blot contained J6 RNA, but this was later discovered to be almost completely degraded. The species from which the cDNA probe was derived is indicated in brackets for each annexin.

Annexin IV mRNA was faintly detected in A431 and Swiss 3T3 cells, and mRNA for annexins III and V was detected only in A431 cells. The human annexin V cDNA probe did not detect RNA in PC12 cells, contradicting the results of Schlaepfer and Haigler (1990) who found that although annexin V was expressed at very low levels in undifferentiated cells it was induced five-fold by NGF treatment. This suggests that hybridisation of the human cDNA probe with annexin V mRNA from other species is weak and therefore may not detect annexin V in the rat and murine cell lines.

Annexin VII mRNA was expressed in A431, RBL-2H3, Swiss 3T3 and PC12 cells. A431 cells clearly contain two transcripts, which may represent the two alternative splice forms known to exist, although this is unlikely since these differ in size by only 66bp (Magendzo *et al.*, 1991). The larger, less abundant transcript was not detected in the other cell types. Annexin VII mRNA, just detectable in undifferentiated PC12 cells, was strongly induced by NGF treatment to levels comparable to those in RBL-2H3 cells.

Annexin VIII protein has a very limited tissue distribution and has only recently been identified in human tissue. A431s were the only one of the cell lines examined to express it.

(ii) *Western blotting*

To examine annexin expression at the protein level, cell lysates were prepared from A431, J6, PC12 before and after 3-day exposure to NGF, RBL-2H3 and Swiss 3T3 cells. Equal protein loadings of each cell type were subjected to SDS-PAGE, Western blotting and immunoblotting for those annexins for which antisera were available: I, II, V and VI. The antibodies are described in more detail in Materials and Methods. The affinity of cross-species recognition is unknown, therefore the results can only be judged qualitatively and not quantitatively. The results are shown in figure 3.2.

Annexin I expression was detected in A431, RBL-2H3 and Swiss 3T3 cells, in agreement with the Northern blot data, and J6 cells (not included on the Northern blot). This antibody cross-reacts weakly with annexin II (see section 3.4) which can be seen as a faint band migrating with a slightly faster mobility than the annexin I band in the Swiss 3T3 and A431 tracks, and probably accounts for the weak band visible in the

PC12 + NGF track migrating at the same mobility as rat annexin II in RBL-2H3 cells and not rat annexin I. The rat and murine proteins exhibited identical mobility on SDS-PAGE, human annexin I has a slightly higher mobility.

The choice of anti-annexin II antibody was determined by the results in section 3.2 below: the monoclonal anti-annexin II N-terminus antibody HH7 (antibody 4) was used. Annexin II was detected in A431, PC12 before and after differentiation (there is a several-fold increase after differentiation), RBL-2H3 and Swiss 3T3 cells. The rat and murine proteins exhibited slightly lower mobility on SDS-PAGE than human annexin II.

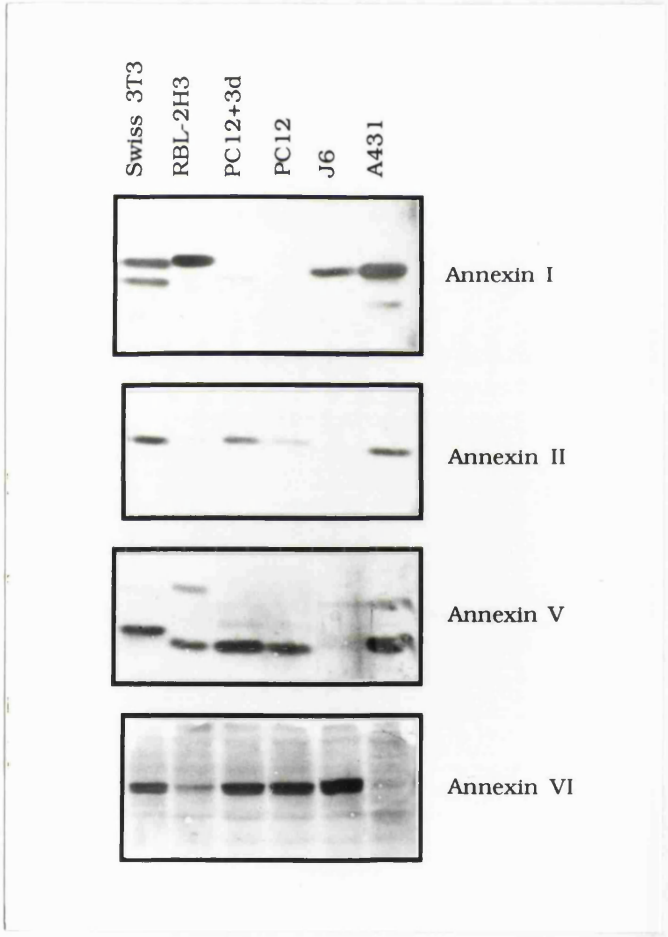


Figure 3.2 *Western blots of protein extracted from A431, PC12 (before and after differentiation induced by exposure to NGF), RBL-2H3 and Swiss 3T3 cells, immunoblotted for annexins I, II, V and VI.*
Gels were loaded with 20µg of protein per track and transferredl to immobilon P after SDS-PAGE.

Annexin V protein was detected in A431 cells and also, in contrast to the Northern blotting data, in PC12 cells before and after differentiation, RBL-2H3 and Swiss 3T3 cells. Annexin V was not detected in J6 cells. It appears that poor cross-species hybridisation between the human cDNA probe and rat and murine mRNAs prevented their detection by Northern blotting. Two bands of different molecular weight (M_r) were detected in A431, RBL-2H3 and Swiss 3T3 cells. PC12 cells appeared to express only the lower M_r form of annexin V, which is induced several-fold by NGF treatment. Two forms of bovine annexin V have been reported that differ in only two amino acids but show an apparent 4kD difference in mobility on SDS-PAGE (Bianchi *et al.*, 1992a). Interestingly the two forms in RBL-2H3 cells differ in mobility to a greater extent than either the human (A431) or murine (Swiss 3T3) protein doublets; and the lower molecular weight forms of rat and murine annexin V migrate on SDS-PAGE at different molecular weights. This suggests that the mobility of this protein is exquisitely sensitive to minor changes in the amino acid sequence.

The characteristic doublet of two alternatively spliced forms of annexin VI can be seen in J6, PC12 cells before and after differentiation, RBL-2H3 and Swiss 3T3 cells. No annexin VI was detected in A431s, in agreement with other workers who have demonstrated that annexin VI is not expressed in A431s (Smythe *et al.*, 1994). While annexins I, II and V exhibited different mobilities on SDS-PAGE depending on the species from which they are derived, annexin VI from all cell types in this study had identical mobility.

3.2 Characterisation of five anti-annexin II antibodies

Annexin II is the focus of this study and several different antisera were available. The names and main features of the five antibodies are listed below (more details are given in Materials and Methods).

Antibody 1 αCal 1-15, polyclonal raised against residues 1-15.

Antibody 2 αp36I, polyclonal raised against whole human protein.

Antibody 3 polyclonal raised against whole porcine protein.

Antibody 4 HH7, monoclonal raised against residues 1-18.

Antibody 5 mono-αp36, monoclonal raised against native whole human protein.

To evaluate any differences in specificity and/or affinity of the five different anti-annexin II antibodies, identical SDS-polyacrylamide gels of equal protein loadings of cell lysates from Swiss 3T3, RBL-2H3, PC12, J6 and A431 cells were Western blotted and immunoblotted with each of the five antibodies. The results of this are shown in figure 3.3 A-E.

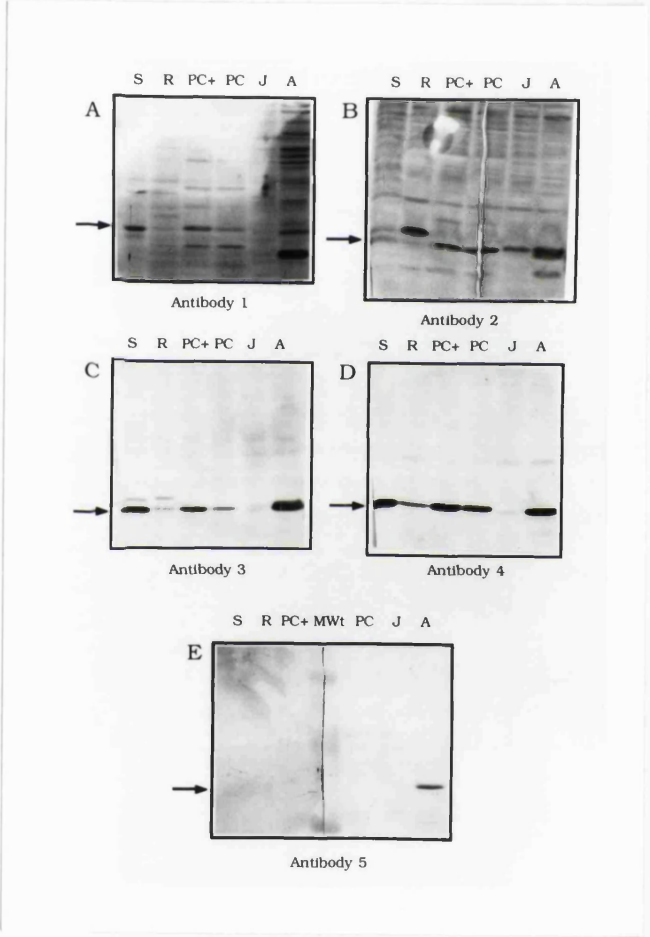


Figure 3.3 *Western blots of protein extracted from A431, PC12 (before and after differentiation induced by exposure to NGF), RBL-2H3 and Swiss 3T3 cells, immunoblotted with five different anti-annexin II antibodies.*
Gels were loaded with 20µg of protein per track, from A431 (A), PC12 before and after differentiation induced by exposure to NGF (PC and PC+), RBL-2H3 (R) and Swiss 3T3 (S) cells. An arrow on the left hand side of each panel denotes the position of annexin II. The track containing the molecular weight markers is marked "MWt" in panel E.

Surprisingly antibody 1, raised against residues 1-15 of the N-terminus which are identical between human, murine and rat annexin II, showed a considerable degree of cross-reactivity with other proteins and only very weakly decorates annexin II in the RBL-2H3 cell lysate, although in PC12, Swiss 3T3 and A431 cells annexin II was recognised with greater affinity over background bands. Antibody 1 recognised a strong band of approximate molecular weight 32kD in the A431 cells of unknown origin. (A band of this mobility was also recognised in A431 cells by antibody 2.)

Antibody 2, although raised against annexin II appeared to recognise annexin I with higher affinity. (This is clearly seen in figures 3.4.) In figure 3.3 antibody 2 detected annexins I and II in Swiss 3T3, RBL-2H3 and A431 cells; annexin II only in PC12 cells; and annexin I in J6 cells. Antibody 2 also recognised a band of approximately 42kD which may be annexin VII, and a doublet of bands of molecular weight 70kD which could be annexin VI, with higher affinity than the background bands. This antibody is polyclonal and may thus recognise epitopes that are conserved between annexin family members.

The polyclonal antibody 3 also cross-reacted with annexin I in those cells that express it (Swiss 3T3, RBL-2H3, J6 and A431s), but not as strongly as it recognised annexin II. Thus the polyclonal antibodies 2 and 3 recognise epitopes common to annexins I and II.

The monoclonal antibody 4, HH7, detected annexin II in all cells that express it with higher affinity than any of the other antibodies tested. This antibody was raised against the N-terminus and is highly specific: it did not recognise annexin I, although it showed a very weak cross-reactivity with a protein band of approximately 45kD in all cell types.

Antibody 5 only detected annexin II expression in A431 cells and therefore appears to be specific for human annexin II. This suggests that this antibody recognises an epitope in the human protein that is poorly conserved in annexin II from other species. This antibody had the lowest affinity of all the antibodies tested, i.e. gave the weakest signal. This may be because it was raised against *native* whole annexin II

protein and therefore may recognise epitope(s) that are altered or lost upon denaturing gel electrophoresis.

A western blot of five gel tracks of RBL-2H3 cell lysates (unequal protein loadings) was cut into pieces and immunoblotted with different antibodies raised against annexins I and II (figure 3.4). Anti-p35I, raised against human placental annexin I, recognises only annexin I in RBL-2H3 cells. The monoclonal antibody 4, HH7, detects only annexin II which runs at a slightly lower molecular wieght. In figure 3.4 it can be seen that antibody 2 recognises annexin I with higher affinity than annexin II, as well as another band of approximately 50kD.

From the results of figures 3.3 and 3.4 it was concluded that HH7, antibody 4, was the best one to use for subsequent analysis of annexin II in RBL-2H3 cells, being of the highest affinity and specificity for rat annexin II.

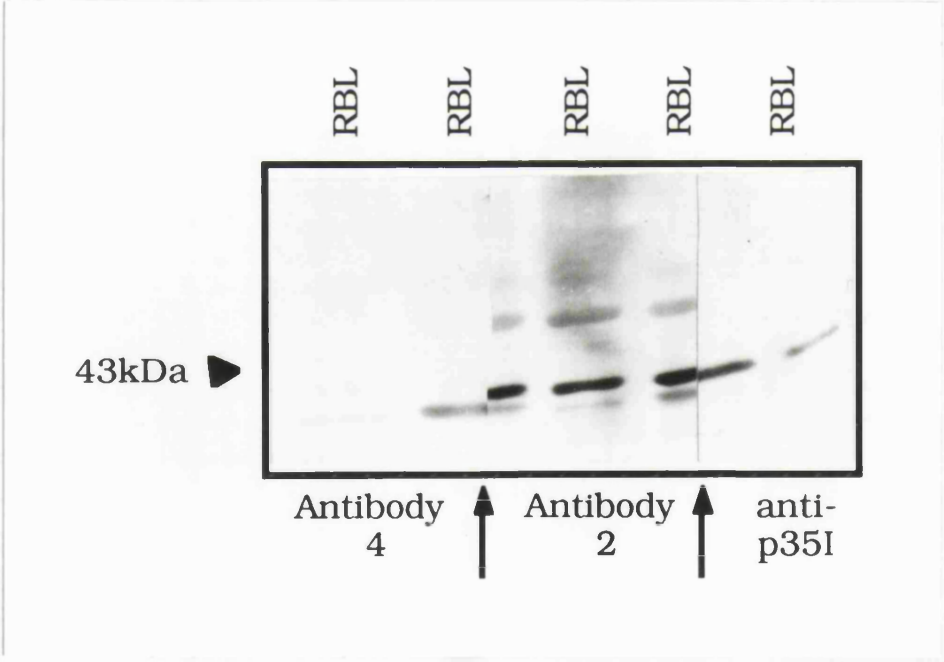


Figure 3.4 *Western blots of RBL-2H3 cell lysates, immunoblotted with anti-annexin II antibodies 2 and 4, and the anti-annexin I antibody anti-p35I.*
The position of the 43kDa molecular weight marker is indicated on the right hand side. Arrows indicate the sites where the Western blot was cut, to produce sections to be immunoblotted with the different antibodies.

3.3 Clonal variation within an RBL-2H3 cell population

To assess the extent to which annexin expression levels vary naturally between cells in a population of RBL-2H3 cells, individual clones were isolated. This was achieved by diluting a trypsinised suspension of cells to a concentration of 1 cell per 200µl, and aliquoting 50µl of this into each well of a 96-well plate. The chances of two cells being deposited in the same well were 2.6% (assuming a Poisson distribution). This resulted in the appearance of colonies in 13 of the wells, several of which grew at different rates. Four clones grew so slowly that they were discarded. The remaining nine clones were cultured in sufficient numbers to be immunoblotted with the available antisera for members of the annexin family: annexins I, II, V and VI.

Within these nine there was considerable variation in growth rate, i.e. in the number of days before the original cell had multiplied to fill a 3 cm dish. Cells that grow at a slower rate have a selective disadvantage and would be expected to be lost from a population of cells that were regularly passaged. This suggests that autocrine stimulation is involved in controlling growth rate in these cells. Presumably in a mixed culture all cells are exposed to the same concentration of extracellular factors. Therefore, as long as they all respond to a similar degree they will all grow at a similar rate. If individual clones produce different quantities of a growth factor then when grown in isolation this will manifest itself in different growth rates. It was beyond the scope of this project to investigate this further.

Four identical SDS-polyacrylamide gels of approximately equal protein loadings of each clone were Western blotted and immunoblotted for annexins I, II, V and VI. The results are shown in figure 3.5.

Some variation was apparent in the annexin I and II levels of different clones, but not more than 20-30% as judged by eye (this has been accentuated by uneven fading of the blots before photographing).The protein levels of annexins V and VI were similar in all clones and the parent population. From these blots it was concluded that there was no significant clonal variation in the expression of these members of the annexin family, and variations in growth rate were not correlated with variations in annexin I, II, V or VI expression.

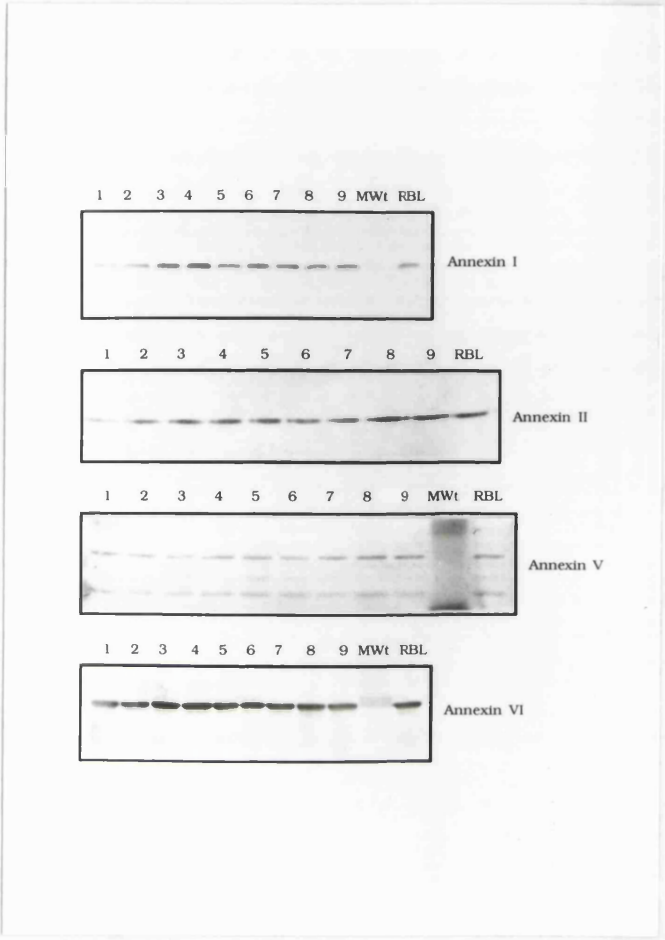


Figure 3.5 *Western blots of RBL-2H3 cell clones, immunoblotted for annexins I, II, V and VI.*
Gels were loaded with 20µg of protein from one of each of the nine clones per track. Only the section of each blot containing the annexin is shown.

3.4 Immunolocalisation of annexin II in RBL-2H3 cells

To examine the intracellular distribution of annexin II in RBL-2H3 cells they were immunostained according to the method of Thiel *et al.* (1992) in a cytoskeletal preservation buffer containing calcium (see Materials and Methods). The antibody HH7, demonstrated to be highly specific in section 3.2, was used for this study. After permeabilisation, cells were incubated with HH7 followed by an FITC-conjugated anti-mouse IgG to visualise the location of the primary antibody. However results with this direct immunolabelling system were poor, so a biotin-avidin amplification system (Streptavidin-TX) was employed. Following incubation with HH7, a biotinylated anti-

mouse IgG was used as the second antibody, the signal was then amplified using a Texas Red avidin-conjugate, many of which can bind to a single biotinylated IgG molecule. This produced the micrographs shown in figure 3.6 B-D.

The immunostaining in figure 3.6 B and C reveals that in most cells annexin II is predominantly located around the cell periphery and appears to be closely associated with the plasma membrane and/or cortical cytoskeleton. This agrees with the work of others who have localised the annexin II tetramer to the sub-plasma membranous cytoskeleton. As well as the peripheral location, occasional areas of punctate immunoreactivity were observed within most cells. The cytoplasmic bodies stained in these cells could be organelles with membrane-bound monomeric annexin II. These structures could be secretory or endosomal vesicles as annexin II has been associated with both. In those cells where processes off the main cell body can be seen, annexin II is diffusely spread within them. This may indicate that the cortical cytoskeleton does not extend along them and that only monomeric annexin II is located here.

There was a large variation in the degree of immunofluorescence among the cell population. The close proximity of cells showing large differences in immunostaining under fluorescence rules out the possibility that this is the result of local variations in antibody concentration or uneven permeabilisation. Therefore, levels of annexin II appear to be dynamic within these cells.

A phase contrast image of RBL-2H3 cells is shown in figure 3.6 A. Note the enlarged cell in the bottom left hand corner. Such cells arise spontaneously in culture from time to time and if isolated will revert to the normal phenotype after a few cell divisions (Michael Beaven, personal communication). Figure 3.6 D contains three of these unusual, enlarged cells. Interestingly, these cells exhibit enhanced immunoreactivity within the cytosol suggesting an elevated concentration of annexin II within the cells as a whole. The immunostaining is not concentrated around the cell periphery but shows more granular staining throughout the cytosol than the normal, smaller cells as well as strong, punctate staining of cytoplasmic bodies. Thus, these cells exhibit an elevated and altered distribution of immunostained annexin II. The negative controls, without either the first or second antibody, were completely blank (data not shown).

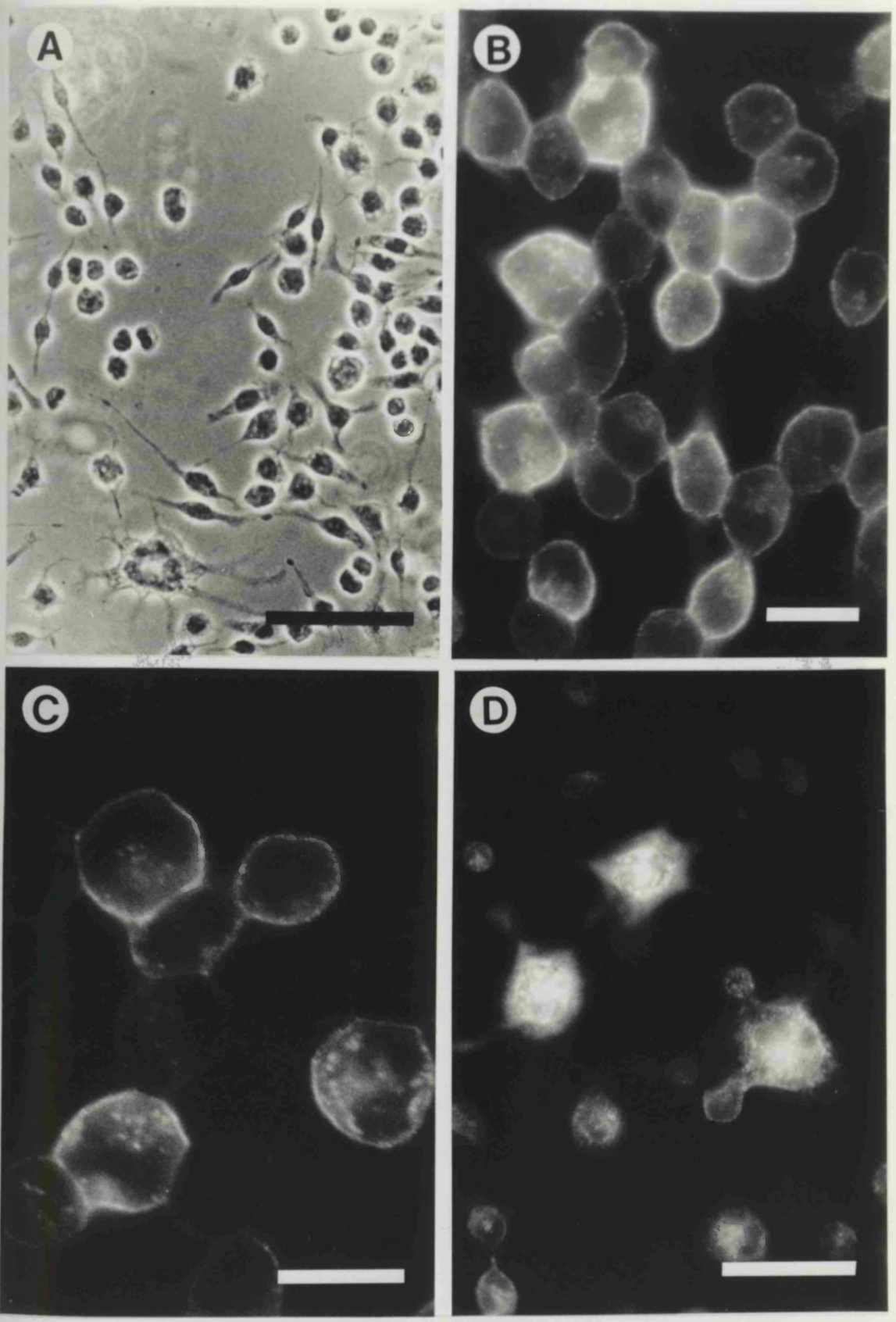


Figure 3.6 *RBL-2H3 cells immunolabelled for annexin II.*
A) Low power phase micrograph of RBL-2H3 cells growing in culture. Scale bar = 100μm. B) Annexin II immunofluorescent-labelled RBL-2H3 cells showing the predominantly cortical distribution of staining. Many cells also exhibit punctate staining within the cytosol. Scale bar = 20μm. C) Higher power micrograph of immunofluorescent cells from panel B. Scale bar = 20μm. D) Unusually large cells with altered annexin II distribution. Scale bar = 100μm.

The assumption is made that HH7 can recognise the N-terminus of annexin II when it is complexed with p11, i.e. that both monomeric and tetrameric annexin II are detected by this antibody. The fact that part of the staining pattern coincides with the reported location of tetrameric annexin II suggests that this is so, but it is open to doubt since binding of p11 is known to alter the conformation of the N-terminus and will cover some of the N-terminal epitopes. If it is the case that the antibody only recognises the monomer then the micrographs in figure 3.6 indicate that some monomeric annexin II is also located at the cell periphery, presumably bound to the inner surface of the plasma membrane since p11 binding is required for attachment to the cytoskeleton (Thiel *et al.*, 1992).

3.5 Secretion from RBL-2H3 cells

Secretion from RBL-2H3 cells has previously been well characterised (Beaven *et al.*, 1987 and references therein). However since this cell line has been cultured for many years in different laboratories and clonal variation is known to have arisen, the secretory characteristics of these cells were analysed as described in Chapter 2.3.

Previous experiments have shown that maximal exocytosis from permeabilised RBL-2H3 cells is achieved at a calcium concentration of approximately pCa5. To determine the time course of hexosaminidase release, exocytosis was triggered from permeabilised cells by pCa5.25 over a range of GTPγS concentrations (figure 3.7).

Secretion from RBL-2H3 cells began within the first couple of minutes of exposure to Ca^{2+} and GTPγS , and was virtually complete in 20 minutes. The small amount of hexosaminidase release after this time was probably due to disintegration of cells, rather than genuine exocytosis,as the rate of release after 20 minutes is about the same for all GTPγS concentrations. In subsequent assays, secretion was measured after 20 minutes.

Secretion in response to a range of Ca^{2+} and GTPγS concentrations was characterised (figure 3.8). Secretion increases as calcium and GTPγS concentrations are increased. At very low calcium (pCa7) there was no exocytosis above basal release from intact cells, even with 100μM GTPγS. At low GTPγS there was a small amount of

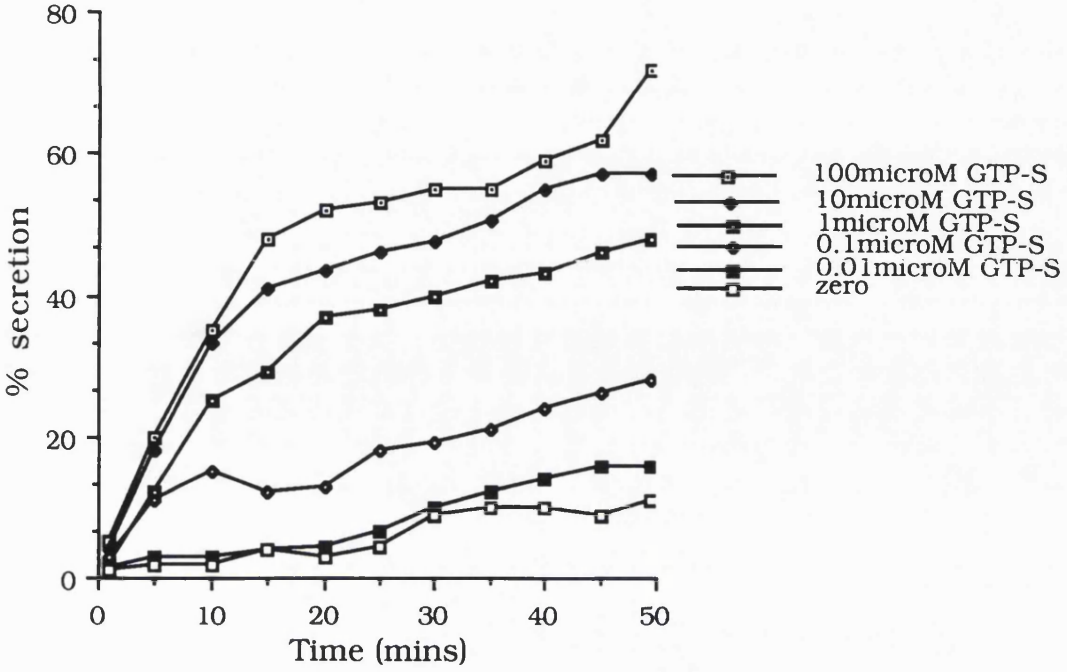


Figure 3.7 *Time course for secretion from RBL-2H3 cells at pCa5.25.*
Cells were permeabilised with SL-O and incubated in solutions containing the indicated concentrations of GTPγS (GTP-S) and 5mM ATP. Secretion was stopped at the time points indicated by the addition of EDTA. Each time point was done in triplicate and this is the result of a typical experiment.

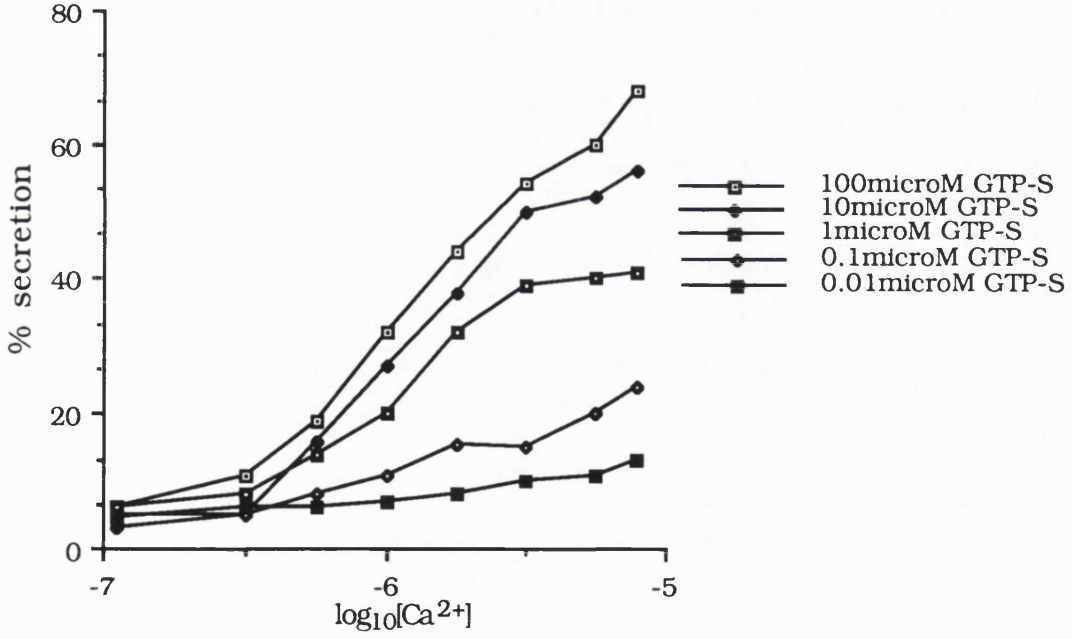


Figure 3.8 *Secretion from RBL-2H3 cells in response to the secretagogues Ca^{2+} and GTPγS*
Cells were permeabilised with SL-O and incubated in solutions containing the indicated concentrations of calcium ($\log_{10}[Ca^{2+}]$) and GTPγS (microM GTP-S). Secretion was stopped after 20 minutes by the addition of EDTA. Each time point was done in triplicate and this is the result of a typical experiment.

hexosaminidase release with [Ca^{2+}] above pCa6. Figure 3.7 shows that at high [Ca^{2+}] less than 5% secretion over basal can occur in the absence of GTPγS. This small amount may be possible because GTP is produced from ATP in the permeabilising medium by nucleoside diphosphate-kinase action on GDP.

Unlike mast cells RBL-2H3 cells have an absolute requirement for ATP to exocytose. All of the above experiments were done in the presence of 5mM ATP. Figure 3.9 shows a typical ATP dose response graph.

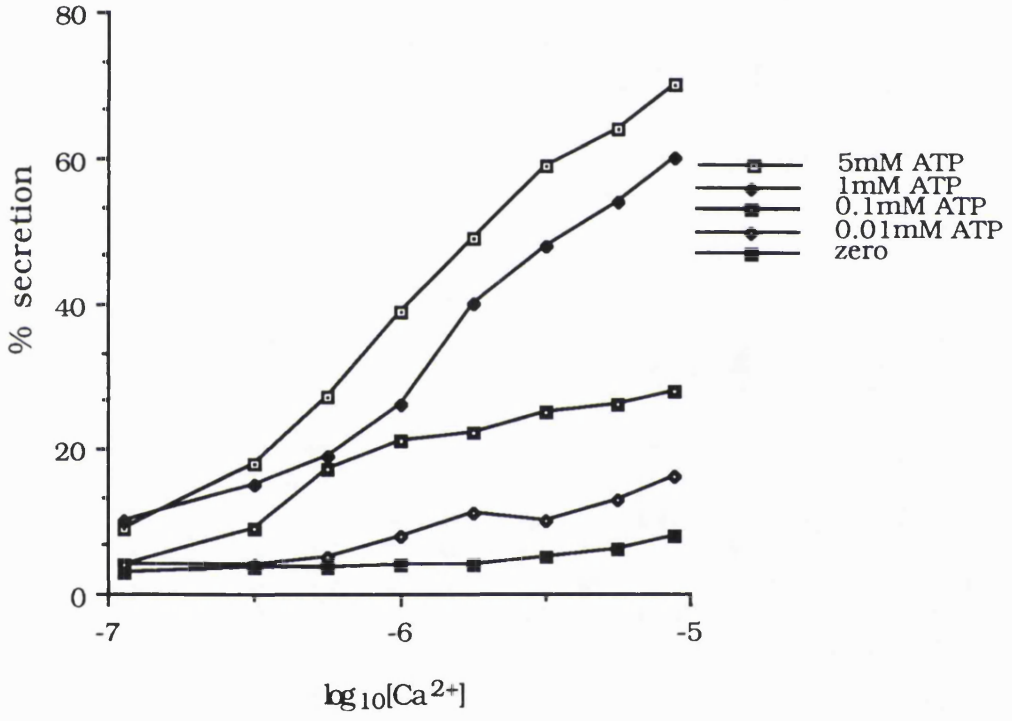


Figure 3.9 *ATP dose response graph for RBL-2H3 cells at 10μM* GTPγS
Cells were permeabilised with SL-O and incubated in solutions containing the indicated concentrations of calcium and ATP. Secretion was stopped after 20 minutes by the addition of EDTA. Each time point was done in triplicate and this is the result of a typical experiment.

The maximal response was elicited in the presence of 5mM ATP, although the response was almost saturated by 1mM ATP. At high [Ca^{2+}] very little exocytosis occurred in the absence of ATP, and this was probably dependent on residual triphosphate nucleotides rather than being true ATP-independent secretion. The

requirement for ATP even in the presence of 10μM GTPγS implies that a phosphorylation event is essential for secretion to proceed (rather than indirect activation of a GTP-binding protein).

3.6 Annexin II leakage from RBL-2H3 cells permeabilised with Streptolysin-O and run-down of the secretory response

(i) *Does annexin II leak out of permeabilised RBL-2H3 cells?*

Workers studying other secretory cell types have found that annexin II, along with other cytosolic proteins, leaks from permeabilised cells (Wu and Wagner, 1991). Loss of cellular proteins causes a reduction in cells' ability to secrete in response to Ca^{2+} and GTPγS. This is known as run-down. Supplying annexin II in the permeabilising medium has been shown to restore responsiveness to chromaffin cells that have been allowed to run down (Ali *et al.*, 1989b). To investigate loss of annexin II from SL-O-permeabilised RBL-2H3 cells, ten 30mm dishes of RBL-2H3 cells were permeabilised for various lengths of time. The supernatant from each dish was precipitated with acetone and the protein pellet resuspended in SDS-PAGE sample buffer for electrophoresis, Western blotting and immunoblotting with anti-annexin II antibody HH7 and the anti-annexin I antibody anti-p35I. The results of this are shown in figure 3.10.

A small amount of annexins I and II was present in the supernatant from intact cells in the presence and absence of calcium. However since more appears in the presence of calcium than in its absence (when a cell surface pool would be expected to be released) the protein detected is probably the result of cell damage and not genuine release of annexins I and II from the cell surface.

Annexins I and II were lost from permeabilised cells regardless of the calcium concentration of the permeabilising solution. The last time point in each case shows less protein released than the preceding one; this could be the result of degradation of the annexins by cellular proteases released from internal organelles by the permeabilising agent. Slightly more annexin II leaks from permeabilised cells in low calcium (EGTA) than in pCa5, this would be expected as in the presence of calcium annexin II should bind to cellular membranes. From the last three tracks on each blot it can be

seen that a little less than half the annexin II, and probably more than half of the annexin I is left in the cells after 20 minutes of permeabilisation in either EGTA or pCa5. This indicates that both annexins have a calcium-insensitive cellular pool, which remains in the cell (although a proportion of what remains in the cell will be cytosolic annexin II in equilibrium with that outside). Annexin II that remains in cells permeabilised in EGTA probably comprises the calcium-independent cytoskeletally-attached tetrameric annexin II and calcium-independent membrane-bound annexin II that is known to exist (Emans *et al.*, 1993). Tetrameric annexin II should remain, unless the cytoskeleton to which it is attached starts to disintegrate. Most of the monomeric, cytosolic annexin II loss occurs in the first 10 minutes. Annexin I loss occurs with similar kinetics to annexin II and a calcium-independent attached pool remains in the cell after permeabilisation in EGTA.

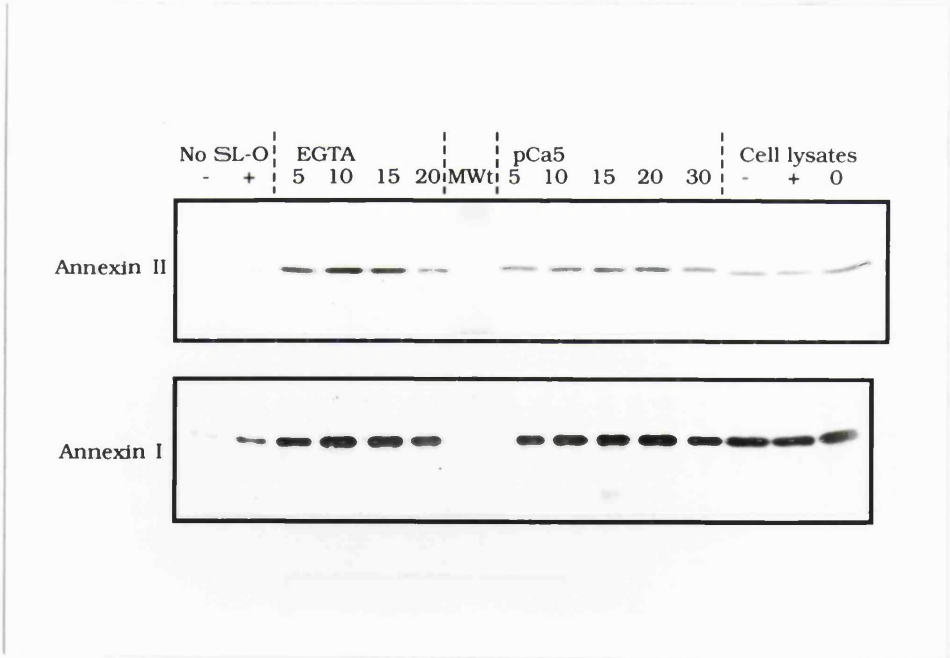


Figure 3.10 *Annexin leakage from permeabilised RBL-2H3 cells*
The leakage of annexins I and II from RBL-2H3 cells permeabilised with SL-O was monitored by immunoblotting cell supernatant with HH7 or anti-p35I. Supernatant was removed at the time points indicated in minutes from separate dishes of cells permeabilised in the presence of EGTA or pCa5. The first two tracks of each blot contain supernatant from intact cells incubated for 20 minutes in EGTA (-) or pCa5 (+). The last three tracks of each blot contain cell lysates from cells permeabilised for 20 minutes in EGTA (-) or pCa5 (+), or intact control cells (0).

(ii) Run-down of secretion

To investigate whether loss of secretory capability was associated with the established leakage of annexin II from permeabilised cells, RBL-2H3 cells, and rat peritoneal mast cells for comparison, were treated with SL-O as described in Materials and Methods and stimulated to secrete at 0, 10, 20 and, for RBL-2H3 cells, 30 minutes after permeabilisation (figure 3.11 A and B). Cells were permeabilised in the buffer used for secretion experiments which for RBL-2H3, but not mast cells, contained 5mM ATP.

After 10 minutes of permeabilisation the ability of mast cells to respond even to high levels of Ca^{2+} and GTPγS was greatly reduced. After 20 minutes virtually no secretion was detectable. RBL-2H3 cells behaved differently: they showed only an approximate 20% reduction in secretory capability even after 30 minutes permeabilisation. Part of this retention of secretory capability (compared to mast cells) may be due to maintainance of a phosphorylation state since it has been shown that provision of ATP (which mast cells do not require if stimulated to secrete immediately after permeabilisation) to mast cells can partially maintain their responsiveness to secretagogues for up to 10-15 minutes after permeabilisation (Howell *et al.*, 1989). The ability of RBL-2H3 cells to secrete over 75% of normal after 30 minutes permeabilisation suggests that cytosolic proteins are not necessary for exocytosis from these cells.

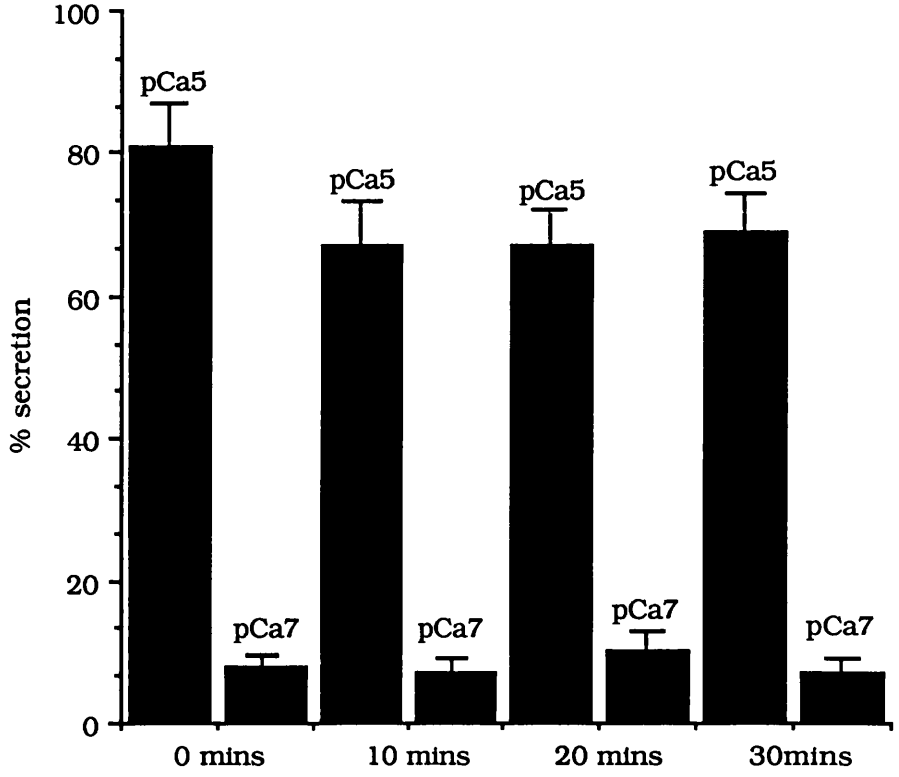
3.7 Discussion

3.7.1 Annexin expression in RBL-2H3 and other cell types

Annexins I, II and VII were detected in RBL-2H3 cells at the mRNA level, and annexins I, II, V and VI were detected at the protein level. The detection of those annexins most strongly implicated in exocytosis (i.e. annexins I, II, VI and VII) is consistent with any one of them being involved in this process.

The results from PC12 cells are interesting because exposure to nerve growth factor causes these cells to differentiate into a secretory cell line, and presumably involves the induction of all the components of the secretory pathway, as well as those associated with other functions of the differentiated cells. Annexin II and annexin VII

A



B

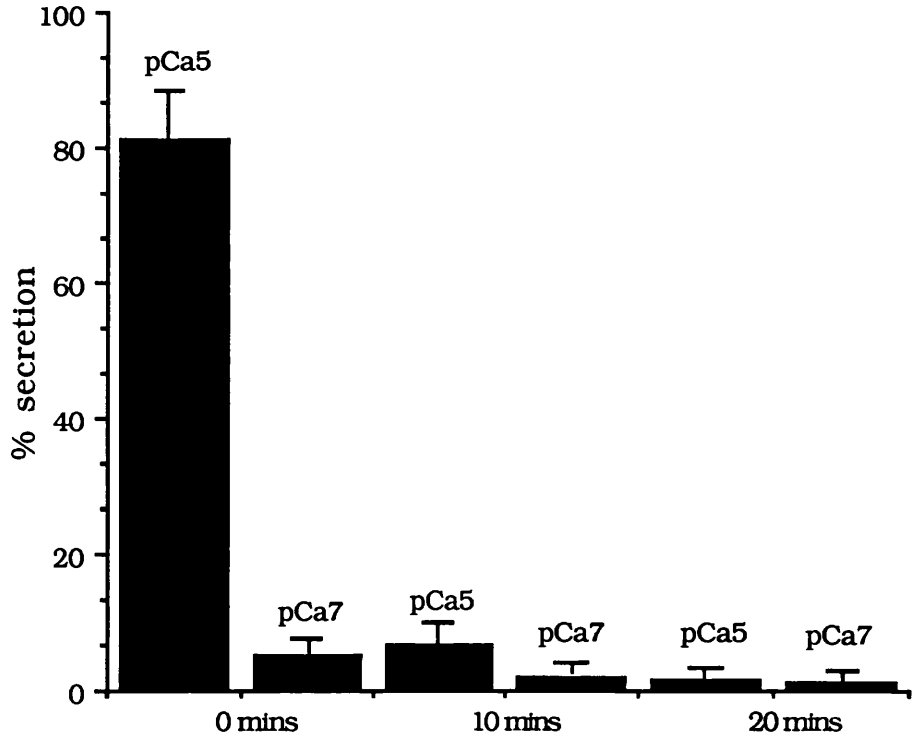


Figure 3.11 *Secretion from permeabilised RBL-2H3 and mast cells allowed to run down before stimulation*
Cells were permeabilised with SL-O and incubated at 37°C for 0, 10, 20 or 30 minutes before stimulation with 10μM GTPγS and either pCa5 or pCa7. Secretion was stopped 20 minutes after stimulation for RBL-2H3 cells and 10 minutes after stimulation for mast cells. A) Secretion from RBL-2H3 cells. B) Secretion from mast cells. Each bar is the mean of 8 samples.

mRNA were strongly induced in differentiated PC12 cells, which is consistent with either or both annexins II and VII being necessary for exocytosis. The absence of annexin II from undifferentiated PC12 cells indicates that its function is not essential to the cells in their undifferentiated state, and is therefore likely to be associated with specialised cellular machinery of the differentiated phenotype such as the secretory apparatus. Annexin I mRNA was not detected in PC12 cells and did not appear upon differentiation (the cDNA probe was from the same species). The absence of annexin I makes it unlikely that this annexin is involved in exocytosis, at least in neuroendocrine cells. The level of expression of annexin VI did not change upon PC12 cell differentiation which suggests that it functions in both the undifferentiated and differentiated cell.

A431 cells express seven of the eight members of the annexin family screened for. This cell line is highly transformed and appears to have switched on almost all of the annexin genes. Although many genes are probably deregulated in A431 cells, several annexins have been implicated in growth control and it is tempting to speculate that they are contributory elements in the transformation event.

Quiescent Swiss 3T3 cells did not express annexin II mRNA, but annexin II protein was detected in serum-stimulated Swiss 3T3 cells. This agrees with the results of Keutzer and Hirschhorn (1990) who previously identified annexin II as a growth-regulated gene in this cell type.

3.7.2 The different specificities of the anti-annexin II antibodies

Five anti-annexin II antibodies have been characterised. Three were of low specificity and cross-reacted with other annexins. Two were highly specific for annexin II, but one of these was of low affinity and was species-specific for human annexin II. Therefore HH7, a monoclonal antibody raised against the N-terminus of annexin II, due to its specificity and high affinity, was chosen for use in further studies with rat annexin II.

The weakness of the αCal 1-15 cross-reaction with annexin II from RBL-2H3 cells raises the possibility that there is an epitope in the N-terminus that is disturbed in

these cells. This antibody strongly cross reacts with a 30-32kD protein in A431 cells which appears to have a common motif.

3.7.3 Immunolocalisation

Immunolocalisation of annexin II in RBL-2H3 cells showed punctate and more diffuse staining in the cytosol, as well as cortical staining. Other workers have shown that the annexin II tetramer is associated with the cytoskeleton just under the plasma membrane, which probably accounts for the cortical staining. Annexin II has been reported to be associated with both early endosomes and secretory granules in other cell types: the punctate staining in the cytosol of RBL-2H3 cells seen here may therefore be endocytic or secretory granules.

There is a high degree of variation in the intensity of immunostaining between adjacent cells which cannot be attributed to staining problems. Rather than perhaps reflecting permanent differences between cells which were not observed when investigating clonal variation between RBL-2H3 cells (section 3.3), this may indicate that annexin II levels vary normally during the cell cycle. The oversized cells that appear occasionally in culture expressed much larger amounts of annexin II than normal sized cells, and showed an altered distribution, the increased expression of this growth-regulated gene may be relevant to their large size (Keutzer and Hirschhorn, 1990). The change in cellular localisation of annexin II in these cells suggests that p11 expression has not been increased to the same extent as that of annexin II (otherwise one would expect annexin II to form tetramers with p11 and attach to the cytoskeleton). This may reflect different cellular functions for the monomeric and tetrameric forms of annexin II, with the monomeric function being particularly important in these large cells.

3.7.4 Run-down of secretion from RBL-2H3 cells and loss of annexin II

The strain of RBL-2H3 cells in this laboratory was shown to have secretory characteristics similar to those published. Secretion is triggered by Ca^{2+} and GTPγS, but

requires ATP (probably to phosphorylate a protein involved in the secretion process). Permeabilisation of up to 30 minutes before addition of secretagogues results in less than a 25% reduction in secretion. Although annexin II was shown to leak from permeabilised RBL-2H3 cells, loss of annexin II was not associated with secretory run-down. However this does not rule out a role for annexin II in secretion since approximately half the annexin II remains in cells after 20 minutes of permeabilisation and can be assumed not to have become rate-limiting for secretion.

A large proportion of annexins I and II remain in the cell when permeabilised in the presence of very low calcium. This indicates that there is a substantial pool of calcium-independent bound annexin I and II. In the case of annexin II this will include the cytoskeletally-attached annexin II tetramer as well as any protein bound to membranes independently of calcium. In the presence of pCa5, annexin II would be expected to bind to cellular membranes and so it is surprising that so much annexin II (and annexin I) is lost from cells at this calcium concentration. This may indicate that cells normally employ a calcium-dependent modification (phosphorylation for example) of annexins I and II to prevent them binding non-specifically to internal cell membranes when intracellular calcium levels are raised.

Chapter 4

Cloning and sequencing rat annexin II

4.1 Isolation of annexin II clones from an RBL-2H3 cell cDNA library

In a permeabilised cell system some and perhaps many of the proteins which contribute to exocytosis will be lost from the cytosol. Annexin II clearly cannot be solely responsible for exocytosis so run-down studies in such systems (Ali *et al.*, 1989b; Wu and Wagner, 1991) are difficult to interpret. In an attempt to examine the effect of loss of annexin II in an otherwise intact system, the secretory cell line RBL-2H3 was chosen to be transfected with an expression plasmid containing antisense annexin II cDNA. This technique should selectively reduce expression of annexin II in these cells and allow the effect of loss of annexin II to be considered in isolation from other components of the secretory pathway. To maximise the efficiency and specificity of hybridisation between the antisense RNA and the endogenous mRNA it is important to use cDNA from the same species. Annexin II has been cloned from several mammals but not rat. Therefore, for studies in a rat cell line, it was first necessary to clone rat annexin II cDNA.

Two RBL-2H3 cDNA libraries, one constructed in the plasmid pcDNAI and the other in bacteriophage λ, were screened at low stringency with a murine annexin II cDNA probe as described in Materials and Methods. Although no positive clones were obtained from the latter, the pcDNAI library yielded several positive clones on duplicate screens. Twelve of these were chosen for secondary screening with the same probe, of which six produced positive colonies. From each of these an individual colony was picked, and plasmid DNA prepared for gel electrophoresis, Southern blotting and screening with radiolabelled murine annexin II cDNA to confirm the presence of annexin II sequence. The autoradiograph of this is shown in figure 4.1.

Positive signals were detected in tracks 1, 3, 5, 8, 9, and 12. Preliminary restriction enzyme analysis of these plasmids using Hind III and Not I (figure 4.2) to

excise the cDNA insert, indicated that four of these were potentially full length, approximately 1.5kb (clones 1, 8, 9 and 12) and two were partial length, 0.9kb (clones 3 and 5).

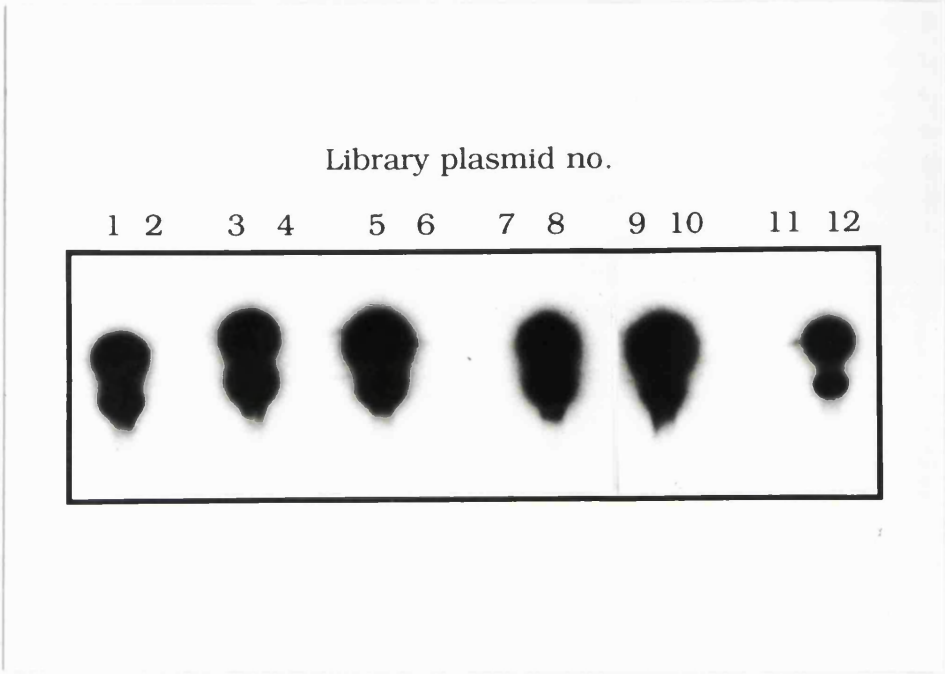


Figure 4.1 *Autoradiograph of cDNA library plasmids screened for rat annexin II.*
Plasmid DNA from 12 cDNA library clones, Southern blotted and probed for the presence of rat annexin II cDNA sequences with a probe derived from murine annexin II.

Figure 4.2 below shows the polylinker region from the cDNA library plasmid, pcDNA I, and indicates the position of restriction sites used for subsequent subcloning.

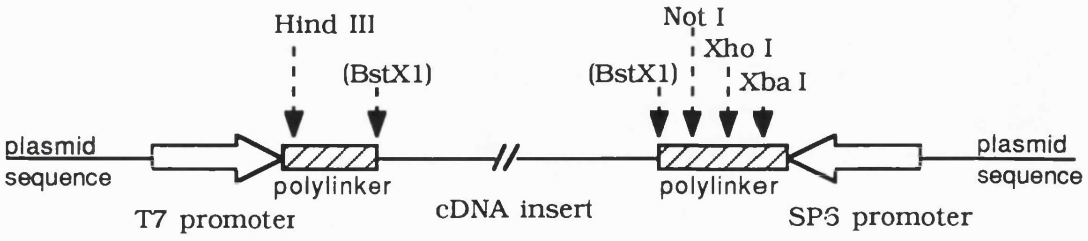


Figure 4.2 *The polylinker region from pcDNA I.*
The Bst XI sites used in construction of the library were not reconstituted and are indicated in parenthesis. The promoter sequences for T7 and SP6 RNA polymerases are represented either side of the polylinker by large arrows.

4.2 Partial sequencing of the six annexin II cDNA clones

The six different rat annexin II-containing plasmids were partially sequenced using the T7 and SP6 priming sites on either side of the cloning site of pcDNA I (see figure 4.2). This allowed approximately 350 bases to be read at each end of the inserted cDNA. Comparing these:

pcDNAI.clone 1 had a 5' untranslated region, a putative translation start site and an N-terminal coding region highly similar to the corresponding murine annexin II sequence. At the 3' end, an identifiable polyadenylation signal and a poly A tail were present. When aligned with the murine sequence the only notable difference was a 6 nucleotide insert in the coding region 45 bases from the probable translation start site.

pcDNAI.clone 3 had a 5' end identical to that of clone 1, but without the 6 nucleotide insert. At the 3' end a short stretch of 23 bases approximately 90 bases upstream of the T7 promoter were identical to residues 765-787 of the complete sequence of clone 1 determined in section 4.3 (located using the DNA handling software DNA Strider), but the surrounding sequence was unrelated. This anomalous clone, which was less than 1kb in length, had presumably become rearranged during cloning and isolation, and was not further investigated.

pcDNAI.clone 5 was identical to clone 1 at the 5' end except that it did not contain the first 14 bases of the 5' untranslated region or the 6-nucleotide insert. The sequence read from the 3' end had 19 unrelated bases followed by a stretch identical to residues 726-830 of clone 1, followed by further unrelated sequence. This clone, which was less than 1kb in length, was also assumed to have undergone rearrangement during the production of the library, and was not investigated further.

pcDNAI.clone 8 was identical to pcDNAI.clone 1 at the 3' end, but lacked approximately 230 nucleotides at the 5' end when compared to the complete sequence of pcDNA I.clone 1 (see section 4.3). This clone probably arose as a result of premature termination by reverse transcriptase during construction of the cDNA library.

pcDNAI.clone 9 was identical to pcDNAI.clone 1 at its 3' end but lacked 135 nucleotides at the 5' end. This clone was also presumed to be a premature termination product.

pcDNAI.clone 12 was identical to pcDNA I.clone 1 at the 5' and the 3' ends except that it

did not contain the extra 6 nucleotides.

This information is represented in diagrammatic form in figure 4.3.

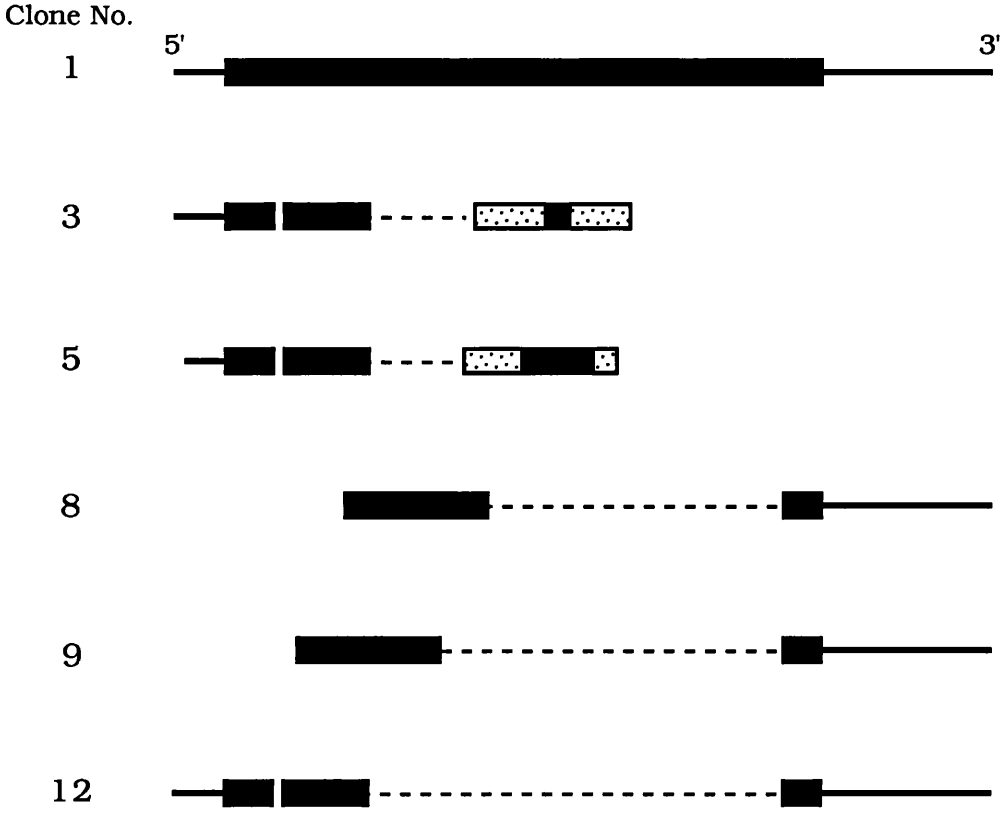


Figure 4.3 *Alignment of the sequenced regions of the six annexin II clones isolated from an RBL-2H3 cDNA library*
Untranslated sequence is represented by thin black line; translated sequence is represented by thick black line; unsequenced portions are represented by dotted line; DNA which was not identified in pcDNAI.clone 1 is represented by dot-filled boxes.

The section of the sequencing gel showing the region including the 6-nucleotide insert in pcDNA1.clone1 is shown in figure 4.4 with the same region from pcDNAI.clone 12 for comparison.

4.3 Complete sequencing of a full-length rat annexin II cDNA clone

To complete the sequencing of rat annexin II cDNA, fragments of pcDNA1.clone 1 were subcloned into pBluescript SK^+. As restriction sites within the cDNA were revealed by sequencing, these were used to subclone different fragments into pBluescript

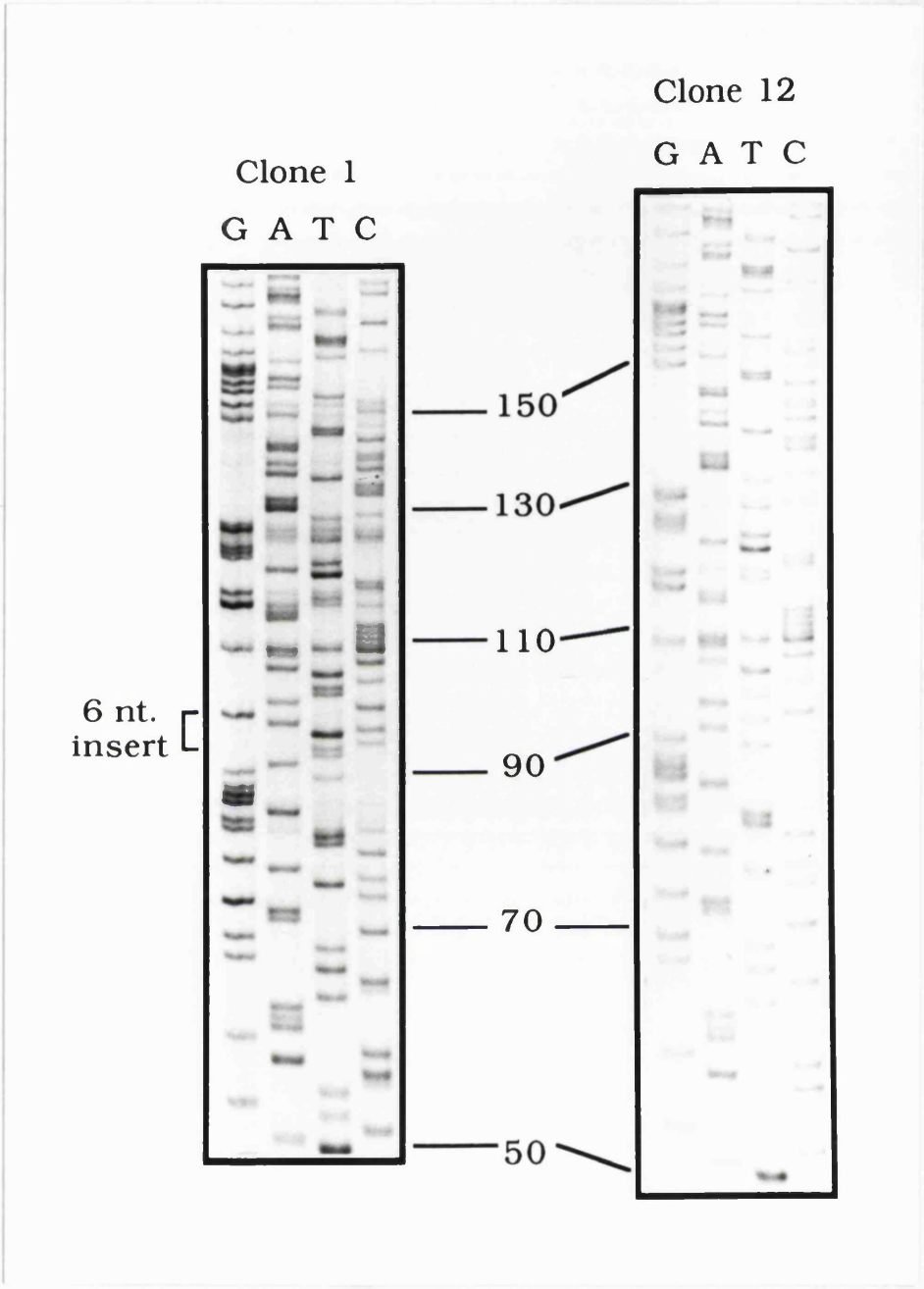


Figure 4.4 *Sequencing gel showing the region of pcDNAI.clone 1 containing the extra six nucleotides*
The same region from clones 1 and 12 are shown for comparison. The location of the extra nucleotides, TCTCAG, is marked beside the panel containing the clone 1 sequence. The number of nucleotides from the start of the cDNA is indicated between the panels.

to continue and facilitate further sequencing. The first fragment cut out of pcDNAI.clone 1 for subcloning extended from the Hind III site in the polylinker at the 5' end to an Xba I site at nucleotide 1127 in clone 1 (see figure 4.2). The fragment was isolated from an agarose gel and subcloned into Hind III-Xba I-cut pBluescript. Sequencing in from the 3' end revealed an Xho I site 230 bases upstream (nt. 897). This was used to produce a Hind III-Xho I fragment from pcDNAI.clone 1, that was ligated into Hind III-Xho I cut pBluescript. To complete the sequencing, an internal Bst XI fragment (nucleotide 192-1271) was blunt-ended, ligated into Sma I-cut pBluescript, and sequenced in from the 5' end. To overcome a stretch of unreadable sequence in the region of nucleotides 360 to 390, a short fragment from the 5' end of annexin II cDNA was cut from pcDNAI.clone 1 using the Bam HI restriction site in the polylinker and Sau3AI at position 439. The fragment was ligated into Bam HI cut pBluescript. Sequencing from the 3' end revealed the remaining nucleotides. The full sequencing strategy is shown in figure 4.5 below.

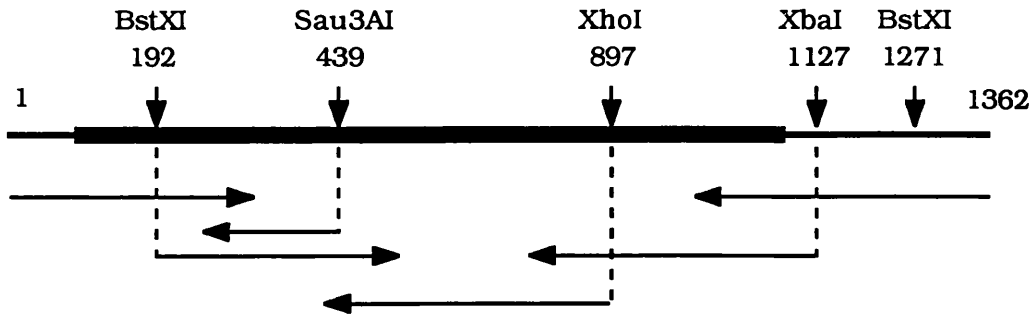


Figure 4.5 *The sequencing strategy for rat annexin II cDNA.* Untranslated portions of the cDNA arerepresented by thin black line; translated regions by thick black line. The position of restriction enzyme sites used to subclone fragments of pcDNA1.clone1 into pBluescript are marked, and the direction and extent of sequencing is indicated by the direction and length of the arrows.

The complete nucleotide sequence of pcDNA.clone 1 is shown in figure 4.6, with the derived amino acid sequence. The cDNA is 1362 nucleotides in length, there are 44base pairs of 5' untranslated sequence and 292 base pairs of 3' untranslated sequence,

including the 18 adenosine residues of the poly A tail. The putative coding region extends from residue 45 to 1070 and is predicted to encode a protein of 340 amino acids (not including the starting methionine). The predicted molecular weight of this protein is 38.74kDa. The designated coding region represents the largest single open-reading frame and is based on its similarity to other mammalian annexin II sequences. In support of this the ATG start codon conforms to the Kozak consensus for a translation initiation site (Kozak, 1986).

The rat cDNA sequence is 93% identical to the murine annexin II cDNA isolated by Saris *et al.* (1986) and can therefore be assumed to be the rat homologue of annexin II. The coding sequences are 94% identical. The 3' untranslated regions are 89% identical with 1 deletion and a block of 5 insertions in the murine sequence. The murine cDNA has only 7 nucleotides of 5' untranslated sequence, compared to the 44 in rat. Of the 64 nucleotide changes between the coding sequences of the rat and murine cDNAs only nine generate a codon for a different amino acid. The protein sequence homology is discussed further in Chapter 6.

After completion of the sequencing, another group published the sequence of a rat annexin II cDNA (Ozaki and Sakiyama, 1993) isolated by differential screening of a cDNA library of a v-*src* transformed rat cell line. Their sequence was 100% identical to that in figure 4.6 except that it did not contain the 6-nucleotide insert or the first two nucleotides at the 5' end.

4.4 Primer extension analysis

In an attempt to confirm the existence of the novel N-terminal variant of annexin II in RBL-2H3 cells, primer extension analysis was performed on poly A^+ RNA isolated from RBL-2H3 cells. In this technique a synthetic oligonucleotide primer is hybridised to a complementary region of mRNA, and reverse transcriptase is employed to synthesise a DNA copy of the mRNA, 5' of the primer. It is preferable to use a primer within 300 bases of the 5' end to minimise premature termination products so this technique is generally used to determine the length of the 5'-untranslated region which can be incomplete in cDNA clones. However, since the 6-nucleotide insert in

```
                ggaggctctctgcaataggtgcccggcccagctttttttcaaa  44
Start
ATGTCTACTGTCCACGAAATCCTGTGCAAGCTCAGCTTGGAGGGTGATTCTCAGCATTCT  104
M  S  T  V  H  E  I  L  C  K  L  S  L  E  G  D  S  Q  H  S    20

ACACCCCCAAGTGCCTATGGGTCGGTCAAACCCTACACCAACTTCGACGCTGAGAGGGAT  164
T  P  P  S  A  Y  G  S  V  K  P  Y  T  N  F  D  A  E  R  D    40

GCTTTGAACATTGAAACAGCAATCAAGACCAAAGGCGTGGACGAGGTCACCATTGTCAAC  224
A  L  N  I  E  T  A  I  K  T  K  G  V  D  E  V  T  I  V  N    60

ATTCTGACTAACCGCAGCAATGCACAGAGGCAGGACATTGCCTTCGCCTACCAGAGGAGG  284
I  L  T  N  R  S  N  A  Q  R  Q  D  I  A  F  A  Y  Q  R  R    80

ACCAAAAAGGAACTGCCATCGGCGATGAAGTCGGCCTTGTCTGGTCACCTGGAGACCGTG  344
T  K  K  E  L  P  S  A  M  K  S  A  L  S  G  H  L  E  T  V   100

ATGTTAGGCCTGTTCAAGACACCTGCTCAGTACGATGCCTCTGAGCTCAAAGCCTCCATG  404
M  L  G  L  L  K  T  P  A  Q  Y  D  A  S  E  L  K  A  S  M   120

AAGGGCCTGGGGACTGATGAGGACTCCCTCATCGAGATCATCTGCTCAAGAACCAACCAG  464
K  G  L  G  T  D  E  D  S  L  I  E  I  I  C  S  R  T  N  Q   140

GAGCTGCAGGAGATTAACCGAGTGTATAAGGAAATGTACAAGACCGATCTGGAGAAGGAC  524
E  L  Q  E  I  N  R  V  Y  K  E  M  Y  K  T  D  L  E  K  D   160

ATCATCTCTGACACATCTGGAGAATTCCGAAAGCTGTTGGTCGCCCTTGCAAAGGGTAAA  584
I  I  S  D  T  S  G  E  F  R  K  L  L  V  A  L  A  K  G  K   180

CGGGCAGAGGATGGTTCTGTTATTGACTACGAGCTGATTGACCAGGATGCCCGGGAGCTC  644
R  A  E  D  G  S  V  I  D  Y  E  L  I  D  Q  D  A  R  E  L   200

TATGATGCTGGGGTGAAGAGGAAAGGAACCGATGTCCCCAAGTGGATCAGCATCATGACT  704
Y  D  A  G  V  K  R  K  G  T  D  V  P  K  W  I  S  I  M  T   220

GAGCGCAGTGTGTGCCACCTCCAGAAAGTGTTCGAAAGGTACAAGAGCTACAGTCCTTAT  764
E  R  S  V  C  H  L  Q  K  V  F  E  R  Y  K  S  Y  S  P  Y   240

GACATGCTGGAGAGCATCAGGAAAGAGGTCAAAGGAGACCTGGAGAACGCCTTCCTGAAC  824
D  M  L  E  S  I  R  K  E  V  K  G  D  L  E  N  A  F  L  N   260

CTGGTTCAGTGCATTCAGAACAAGCCCCTGTACTTTGCTGACCGGCTGTATGACTCCATG  884
L  V  Q  C  I  Q  N  K  P  L  Y  F  A  D  R  L  Y  D  S  M   280

AAGGGCAAGGGGACTCGAGACAAGGTCCTGATTAGAATCATGGTCTCTCGCAGTGAAGTG  944
K  G  K  G  T  R  D  K  V  L  I  R  I  M  V  S  R  S  E  V   300

GACATGTTGAAAATCAGATCTGAATTCAAGAGGAAATATGGCAAATCCCTGTACTACTTC 1004
D  M  L  K  I  R  E  S  F  K  R  K  Y  G  K  S  L  Y  Y  F   320

ATCCAGCAATACACTAAGGGTGACTACCAGAAGGCGCTGCTGTACCTGTGTGCTGGGGAC 1064
I  Q  Q  D  T  K  G  D  Y  Q  K  A  L  L  Y  L  C  G  G  D   340

GACTGAagggcttggcatggtggattgcccagaagtggccctacctgtgccccaacctaa  1124
D  Stop                                                      341
tgttctagagaatcagcctgccactaatggacccctgaactcctccctgtgaagatgacg  1184

acagagctgccgacccatcccccatcttagctgcctttgcctggctttcccctcattctc  1244

tcctttatgccaaagaaatgaacattddagggagttggacgtaccgtctgtgacatgaga  1304

cacttcctcatatgtgtcgtg aataaa ccatttttacttaaaaaaaaaaaaaaaaaa  1362
```

Figure 4.6 *The complete rat annexin II cDNA sequence and deduced amino acid sequence*
Untranslated nucleotides are in lower case; translated nucleotides are in capitals. The putative polyadenylation signal is shown in bold.

pcDNAI.clone1 is close to the 5' end of the coding sequence this technique was applied to detecting its presence in RBL-2H3 mRNA.

4.4.1 Design of antisense oligonucleotide primers

In order to verify the presence and approximate location of the 6-nucleotide insert in the sequence of pcDNAI.clone1 two primers were used: one complementary to a region downstream from the site of the insert, and a second primer complementary to a region upstream of the insert. The primer extension products generated from them needed to be less than about 250 nucleotides in length to enable a difference of 6 nucleotides to be discerned. The primer complementary to a region downstream of the insert would be predicted to yield two primer extension products, derived from mRNA with and without the insert, differing in size by 6 nucleotides. A second primer complementary to a region upstream of the insert should produce a single product corresponding to the length of the 5' untranslated region. The upstream primer was designed to cover the translation start site so any differences between product sizes from the two primers must be a consequence of nucleotides in the coding region.

The position of the primers is shown in figure 4.7 below.

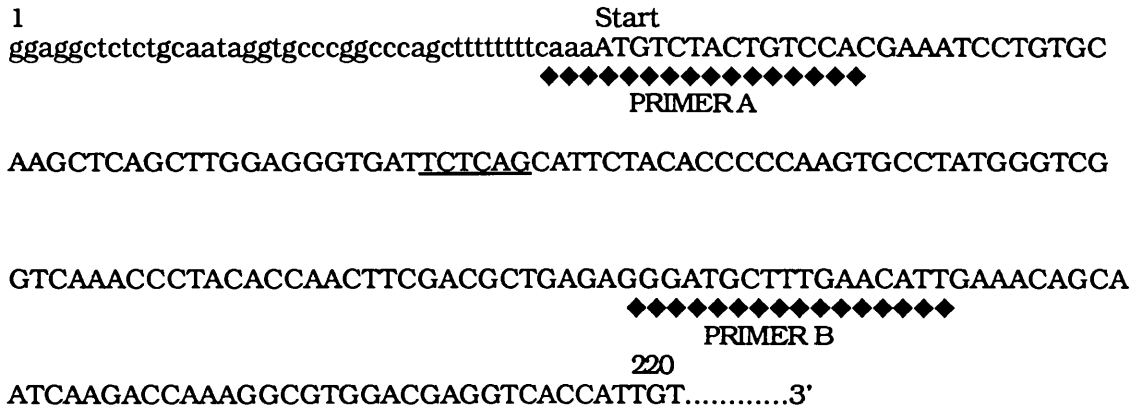


Figure 4.7 *The location of sequences in the rat annexin II cDNA complementary to oligonucleotides used for primer extension analysis* The locations of the nucleotides complementary to the two primers are indicated by solid diamonds. The hexanucleotide insert lies between these two regions and is underlined. Non-coding sequence is shown in lower case and coding sequence in capitals.

The sequence of primer A, a 19-mer, is 5'-GTGGACAGTAGACATTTTG-3'. It is complementary to residues 59 to 41 of the rat cDNA and covers the translation start site. The sequence of primer B, an 18-mer, is 5'-CAATGTTCAAAGCATCCC-3', which is complementary to residues 177 to 160. The region homologous to primer A is identical in rat, mice and human, while the region complementary to primer B is identical in rat and human with one base change in mice. The lengths of the predicted products, calculated from the cDNA sequence, are 59 with primer A and 177 with primer B (or 171 without the hexanucleotide insert). This assumes that the 5' end of the cloned cDNA corresponds to the 5' end of the annexin II mRNA in vivo.

Primer extension analysis was performed using both primers, end-labelled with [^{32}P]-γATP, with mRNA from RBL-2H3, A431, J6 and Swiss 3T3 cells. Figure 4.8 shows the primer extension products from primer B with A431, J6, Swiss 3T3 and RBL-2H3 mRNA, electrophoresed alongside a set of known sequencing reactions (to allow accurate sizing of products) and exposed to X-ray film for two weeks.

Nothing was detected in the J6 and Swiss 3T3 tracks. Three bands were visible in the A431 cell track. The predicted size for the human primer extension product is 176 nucleotides with primer B. The size of the major A431 band was estimated from the sequencing reaction products run alongside the primer extension products' tracks to be 181 nucleotides, slightly larger than predicted from the published human genomic sequence (Spano *et al.*, 1990). This may reflect mobility differences between [^{32}P]-γATP-end-labelled and [^{35}S]-αdCTP labelled DNA, or be a consequence of the different solutions in which the sequencing reactions and primer extensions are performed. The larger product may be the result of an alternatively spliced exon, probably in the non-coding region since a single protein product is detected on Western blotting. In section 3.1 (i), the Northern blot of A431 RNA probed for annexin II shows a second, larger transcript which may account for the higher molecular weight band. The origin of the smaller band is unknown and is most likely to be a premature termination product, possibly caused by secondary structure formation. The RBL-2H3 track contained a single, faint band one base longer than the human primer extension product. Assuming that the A431 band is 176 nucleotides long, the RBL-2H3 cell product is 177 nucleotides

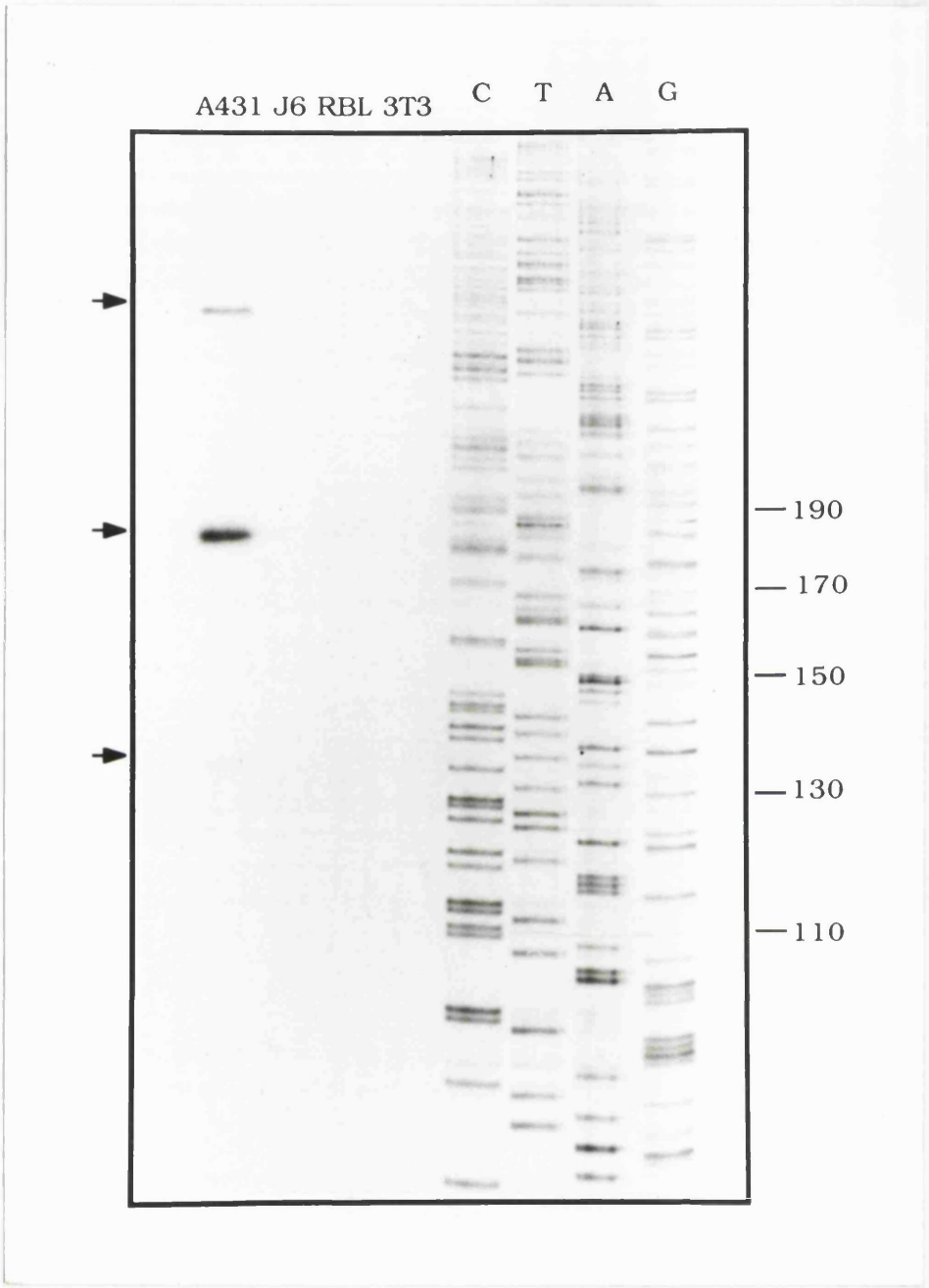


Figure 4.8 *Autoradiograph of primer extension products electrophoresed alongside a set of known sequencing reactions.*
Primer extension products from A431, J6, RBL-2H3 and Swiss 3T3 mRNA using primer B are shown. The number of nucleotides in the sequencing reaction products is indicated on the right hand side.

in length corresponding to the splice form that includes the 6 nucleotide insert. The sequencing data predicted a second band 6 nucleotides shorter but this was not detected.

Several repetitions of this method failed to improve the strength of the signal from RBL-2H3 mRNA, or to achieve any signal with primer A. Attempts were made to increase the signal by adapting the ProMega protocol: instead of end-labelling the oligonucleotide primer, [^{32}P]-αdCTP was included in the primer extension reaction, and the amount of unlabelled dCTP was reduced. Unfortunately this technique produced too many background bands (presumably from non-specific priming and premature termination by reverse transcriptase) to be useful.

4.5 Discussion

The full length annexin II cDNA clone isolated from the RBL-2H3 cDNA library contained a 6 nucleotide insert when aligned with three other clones containing this portion of the cDNA. These six nucleotides are not present in annexin II mRNAs from other mammalian species. The existence of different isoforms of annexins is not unprecedented and the possible origins and implications of this novel N-terminal variant are discussed in Chapter 6.

To show that the hexanucleotide insert was not a cloning artefact, the presence of this mRNA variant in RBL-2H3 cells was examined by primer extension analysis. A band of the size predicted for the primer extension product of this variant was detected on autoradiographs. However the possibility that the 5' end of the cDNA was incomplete and that the extra nucleotides are at the 5' end of the mRNA rather than in the coding region could not be ruled out.

Chapter 5

Transfection of RBL-2H3 cells

5.1 Constructs for transfection in pNV

All studies performed to date investigating a link between annexin II and exocytosis have used permeabilised cells or isolated secretory granules. To examine the effect of alteration of annexin II expression in intact cells, a secretory cell line was chosen for transfection with expression plasmids containing the rat annexin II cDNA in both the sense (to overexpress the protein) and the antisense (to reduce expression of the protein) orientations. The theory underlying the use of an antisense cDNA to reduce expression of a protein is that transcripts from the inserted DNA form a highly stable RNA duplex with the host messenger RNA. This cannot be translated by the ribosomes and is targeted for degradation by RNases in the cell. To maximise the efficiency of hybridisation between the endogenous mRNA and the antisense transcript it is important that they are identically matched. For studies in RBL-2H3 cells it was therefore necessary to clone rat annexin II cDNA (Chapter 4).

pNV, the plasmid originally used for transfection, was a gift from Dafna Bar-Sagi (Cold Spring Harbour) and is described in the Material and Methods section. This was recommended as an expression vector that had been used successfully with RBL-2H3 cells. The plasmid has a single restriction enzyme site, Xho I, at its cloning site, where it was cut, blunt-ended and dephosphorylated for all ligations.

5.1.1 Constructs in pNV containing annexin II cDNA

Full length rat annexin II cDNA, cut from pcDNAI.Clone1 with Hind III and Not I, was blunt-ended and ligated into pNV. DNA from small-scale plasmid preparations from a large number of colonies was electrophoresed in agarose gel, Southern blotted and probed with a labelled rat annexin II cDNA probe. Approximately half of the plasmids gave a positive signal and these were digested with Xho I and Bsc I to determine the

orientation of the insert. pNV contains two Bsc I sites as indicated in figure 5.1 A, and the cDNA contains none. The annexin II cDNA contains a single Xho I site at residue 898; and a second Xho I site was created at the 3' end of the insert when the blunt-end created by filling in the Not I-cut end of the insert was ligated to the filled-in Xho I-cut end of pNV. From figure 5.1 B and C it can be seen that antisense constructs are predicted to produce fragments of approximately 3.9, 1.9, 1.4 and 0.45 kbp, and sense constructs to produce fragments of 4.85, 1.9 and two of 0.45 kbp. From the results of the digest, one plasmid of each orientation was chosen, and large scale preparations made for transfection. The sense construct was named pNV-ANX2 and the antisense construct was named pNV-XNA2.

5.1.2 Constructs in pNV containing annexin I cDNA

For use in control experiments, constructs containing rat annexin I cDNA were created to examine the effects of altering the levels of annexin I in RBL-2H3 cells. A rat annexin I cDNA was excised from pUC 9 (Tamaki *et al.*, 1987) with EcoR I. This was blunt-ended and ligated into pNV as described for annexin II. Due to the relatively high rate of successful ligations with the annexin II cDNA, plasmid DNA from colonies from this ligation were not Southern blotted for the presence of insert but were screened by restriction analysis only.

The restriction map provided with pNV was incomplete, but digestion of pNV with Bgl II and Bsc I yielded three fragments of approximately 4.35, 1.1 and 0.8 kbp. Therefore the Bgl II site must be located in the 1.9 kb fragment between the two Bsc I sites, either 0.8 or 1.1 kb from the end, as indicated in figure 5.2 A. The orientation of the inserts in twenty individual plasmids was determined by digestion with Bgl II. The sizes of fragments generated by Bgl II-digestion of pNV containing the insert in either orientation were calculated using the known position of the Bgl II sites in the annexin I cDNA (the two sites are only 56 bases apart and may be regarded as one for the purposes of mapping). If the insert is in the sense orientation the resulting restriction fragment sizes would be either approximately 2.4 and 5.1, or 2.1 and 5.4 kb, depending on the position of the Bgl II site in the plasmid (see figure 5.2 B).

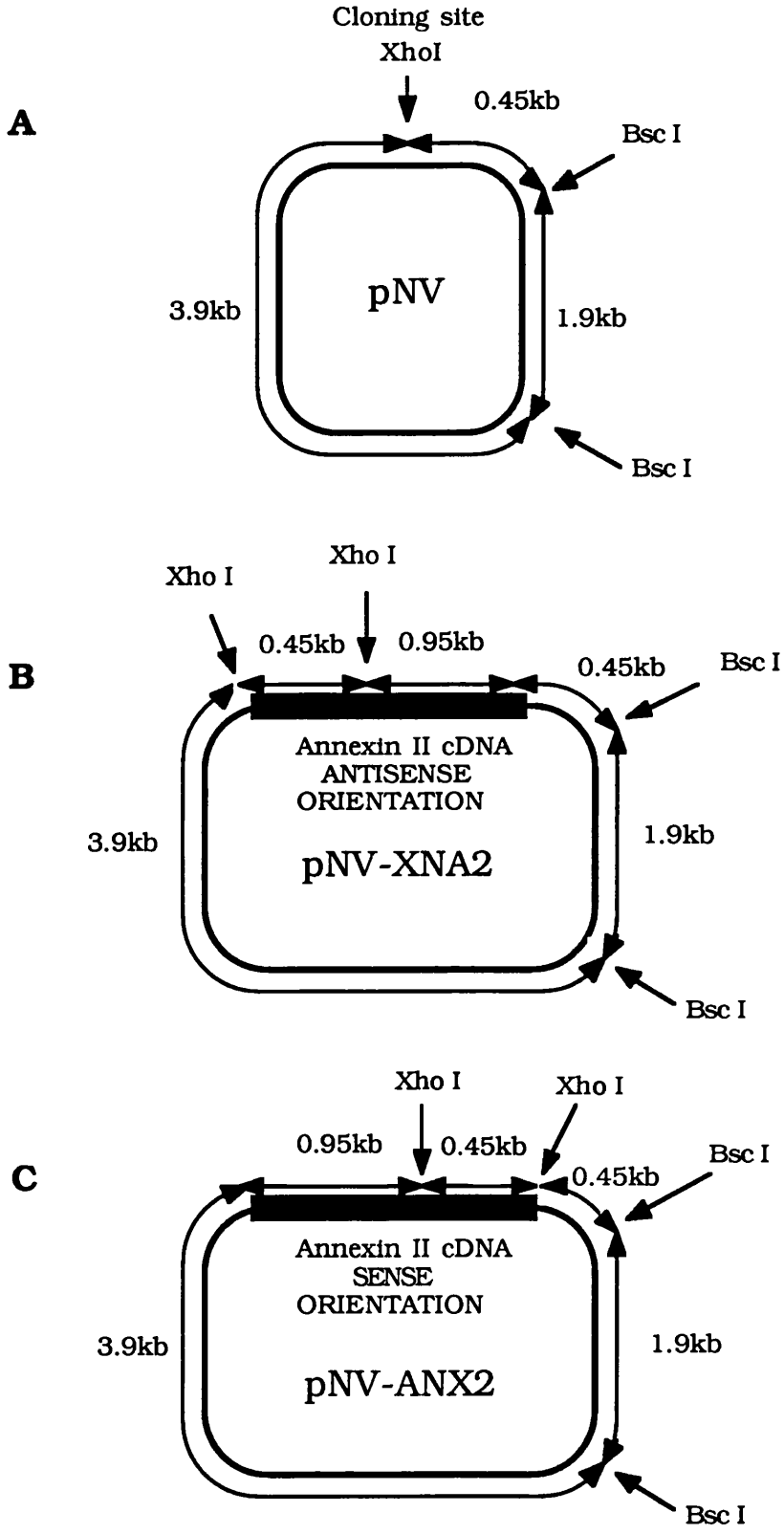


Figure 5.1 *Restriction enzyme sites used to diagnose orientation of the inserted annexin II cDNA in constructs made in pNV*
The position of restriction sites are indicated by arrows and the sizes of portions of DNA are indicated in kb.

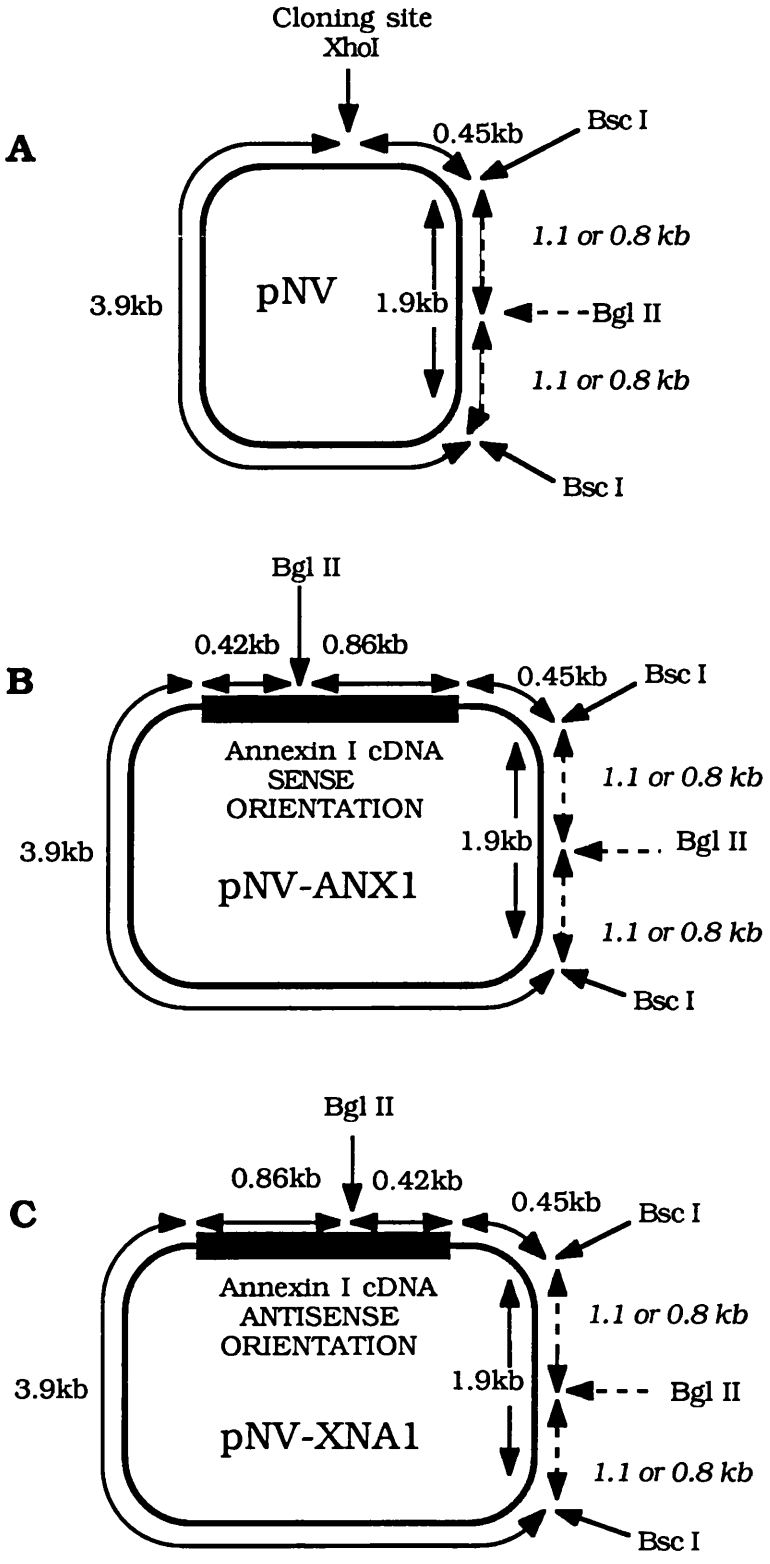


Figure 5.2 *Restriction enzyme sites used to diagnose orientation of the inserted annexin I cDNA in constructs made in pNV*
The position of restriction sites are indicated by arrows and the sizes of portions of DNA are indicated in kb (in italics where uncertain).

If the insert is in the antisense orientation the resulting fragment sizes would be approximately 2.0 and 5.5 or 1.7 and 5.8kb (see figure 5.2 C).

The results of a Bgl II digest on plasmid preps 1-20 indicated that two plasmids contained insert in the sense orientation (fragments of 2.1 and 5.4 kb), and six plasmids contained insert in the antisense orientation (fragments of 1.7 and 5.8kb). One plasmid appeared to contain two concatamerised copies of the cDNA, both in the sense orientation and the remaining plasmids contained no insert. The fragment sizes also confirmed that the Bgl site is 1.25kb from the Xho I site. One plasmid of each orientation was chosen, and a large scale preparation made for transfection. The sense construct was named pNV-ANX1; the antisense construct pNV-XNA1.

5.2 DEAE-dextran- and calcium phosphate-mediated transfection of RBL-2H3 cells

Although diethylamino-ethyl-dextran (DEAE-dextran) mediated transfection is normally used to generate transient transfectants, we had been advised that it could be used for stable transfection of RBL-2H3 cells. Transfection of RBL-2H3 cells was performed as described in Material and Methods. For the first two attempts in which the cells were allowed only 1 day to recover from the dextran treatment before culture in medium containing neomycin, 80% of the cells were dead after a further 2 days, and all were dead after 6 days. For all subsequent attempts, the recovery time before addition of neomycin was extended to 48 hours. In a further effort to improve cell survival following transfection, medium was supplemented with 80μM chloroquine during the 4 hour incubation with DEAE-dextran. However this proved to be moderately cytotoxic: 50% of cells were dead before addition of neomycin, so it was not used again.

It was also considered that plasmid impurity may have compromised the outcome since large scale preparations of plasmids for transfection were purified by PEG precipitation. Plasmids were therefore further purified by centrifugation through a caesium chloride gradient, and the transfections repeated. However this did not improve the success of the technique.

Another possible explanation for the failure to generate stable transfectants was that our RBL-2H3 clone was less robust than clones in other labs. We therefore acquired another strain from Dafna Bar-Sagi (Cold Spring Harbour). Repeating the procedure with these cells (successfully transfected by Dr. Bar-Sagi, personal communication) produced only slightly better results, in that some cells survived up to 12 days in neomycin. It was striking that all cells in different dishes died at the same time. One possible explanation for this is that they had only become transiently transfected and subsequently lost episomally replicating plasmid.

A further possible cause of the problem was that the neomycin resistance gene had become corrupted - this is not unprecedented. To test whether it was functional, pNV, pNV-XNA1 and pNV-XNA2 were used to transfect Swiss 3T3 fibroblasts by the calcium-phosphate technique (see Materials and Methods), a procedure successfully employed by other workers in the laboratory with a different vector. Side by side with this I tried the calcium-phosphate technique with RBL-2H3 cells. Although the transfected RBL-2H3 cells were dead within 6 days of selection in neomycin, stable Swiss 3T3 cell transfectants were established, with all three plasmids, which were still alive after 1 month in neomycin.

Having therefore established the functional integrity of the neomycin resistance gene in Swiss 3T3s, transfection of RBL-2H3 cells by electroporation was attempted, as recommended by Dr. Birgit Helm of Sheffield University (personal communication).

5.3 Transfection of RBL-2H3 cells by electroporation

Several attempts to transfect RBL-2H3 cells were made using this method. Initially approximately $2x10^6$ cells were used each time but since the efficiency of transfection is only 1 in 10^6 at best (Dr. Birgit Helm, personal communication), cell numbers were increased.

Subsequent attempts using this method were more encouraging. Using high cell numbers of approximately $5x10^7$ per attempt, approximately 20 transfected colonies per dish reached the 20-30 cell stage. However in repeated experiments all colonies inexplicably died at the same time. Eventually a faulty incubator was found to be the

source of the problem. Thus, a batch of identically treated pNV-XNA2-transfected RBL-2H3 cells cultured in a different incubator yielded 20-30 stably-transfected colonies per dish. Most of these were isolated and further expanded in 96-well plates prior to transfer to duplicate 30mm dishes. One dish of each of 26 stable pNV-XNA2 transfectants, when confluent, was lysed directly into 250µl of SDS-PAGE sample buffer for analysis of annexin II content. 100µl of each lysate was subjected to SDS-PAGE, Western blotting and immunoblotting with anti-annexin II antibody HH7. The results of these are shown in the top two panels of figure 5.3, the left hand track in each panel is control RBL-2H3 cell lysate. None of these clones showed a significant alteration in the level of expression of annexin II. (Uneven fading of the blots prior to photography has accentuated the differences seen in figure 5.3.)

From further rounds of transfections, 32 pNV-XNA2 colonies and 34 pNV-ANX2 transfected colonies were isolated and cultured. A 3cm dish of each was lysed into 250µl of lysis buffer, centrifuged to pellet cell nuclei and the supernatant mixed with an equal volume of 2xSDS-PAGE sample buffer. 100µl of each sample was electrophoresed and immunoblotted for annexin II with HH7. None of the pNV-XNA2 transfected cells showed a significant reduction in annexin II levels (central three panels of figure 5.3); and none of the pNV-ANX2 transfected clones showed a significant increase in annexin II levels (bottom three panels of figure 5.3).

Although variations in annexin II levels between some of the transfected clones appear to be greater than those observed for naturally occurring clonal variation in section 3.4 none were judged to be significant as cell loadings were approximate, and variations due to the transfected DNA were expected to be greater than those observed.

5.4 Riboprobe analysis of antisense transcripts

Since none of the transfected clones showed a reduction in annexin II protein expression levels, it was important to determine whether antisense message was actually being transcribed. RNA was prepared from four of the pNV-XNA2-transfected clones as well as from the parent RBL cell line. A construct in pBluescript containing the first 900 bp of the annexin II cDNA, up to the Xho I site (see figure 5.4) was used as

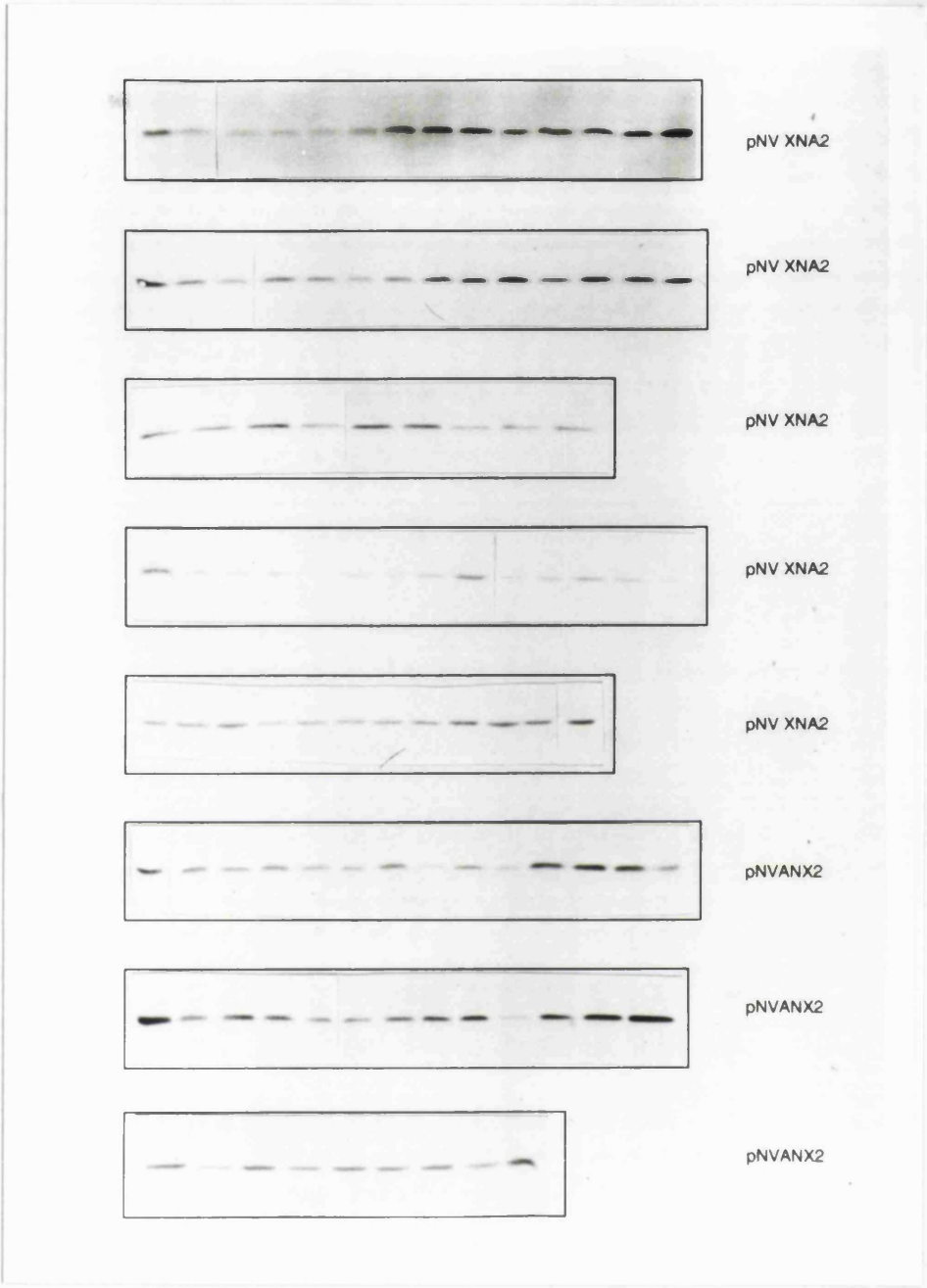


Figure 5.3 *Western blots of RBL-2H3 clones transfected with constructs containing annexin II cDNA in the sense and antisense orientations, immunoblotted for annexin II.*
Clones in the top five panels were transfected with pNV-XNA2 and clones in the bottom three panels were transfected with pNV-ANX2. The left-hand track in each panel contains cell lysate from the parent RBL-2H3 line.

a template for *in vitro* transcription of RNA. Radiolabelled sense strand RNA was produced by small-scale in vitro transcription from the plasmid (linearised with Xho I) using the T3 RNA polymerase promoter located at the 5' end of the insert. The plasmid was linearised with Bam HI, and RNA was transcribed with T7 RNA polymerase to produce unlabelled antisense annexin II RNA to be used as a positive control.

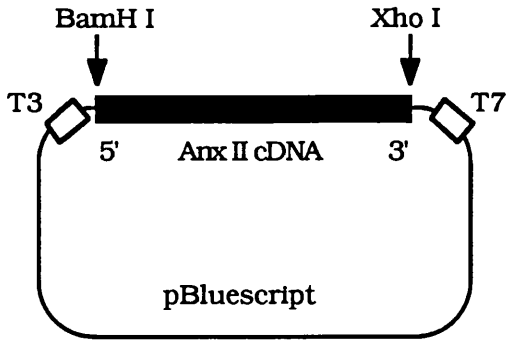


Figure 5.4 *Schematic representation of the template for in vitro transcription of sense and antisense annexin II.*
This figure shows the restriction sites used to linearise the plasmid and the T7 and T3 RNA polymerase promoters (empty boxes) located either side of the inserted 900bp of annexin II cDNA (filled box).

To determine the quantity of positive control RNA synthesised, the incorporation of [^{32}P]-rCTP into trichloroacetic acid (TCA) insoluble material was determined for a portion of the reaction mixture and the amount of RNA synthesised calculated as described in Materials and Methods. 33% of the CTP was incorporated, thus 21.1µg of RNA were synthesised (2.1µg/µl). Various loadings of positive control RNA were subjected to denaturing RNA gel electrophoresis alongside 20µg of RNA from each clone. The gel was Northern blotted and probed with radiolabelled sense strand RNA. The autoradiograph of the riboprobe is shown in figure 5.5.

Even though as little as 1ng of the positive control RNA was detected, there was no signal in any of the RBL-2H3 cell tracks after overnight exposure to X-ray film. Exposure to a phosphorimager plate for 18 hours (not shown) revealed a band in the "100pg" track of positive control RNA but failed to detect anything in the tracks from

RBL-2H3 transfectants. Since 100pg is equivalent to approximately 1 copy per cell (Dr. P. Smith, personal communication) it was apparent that little or no transcription of the antisense orientation annexin II cDNA was occurring in any of these clones.

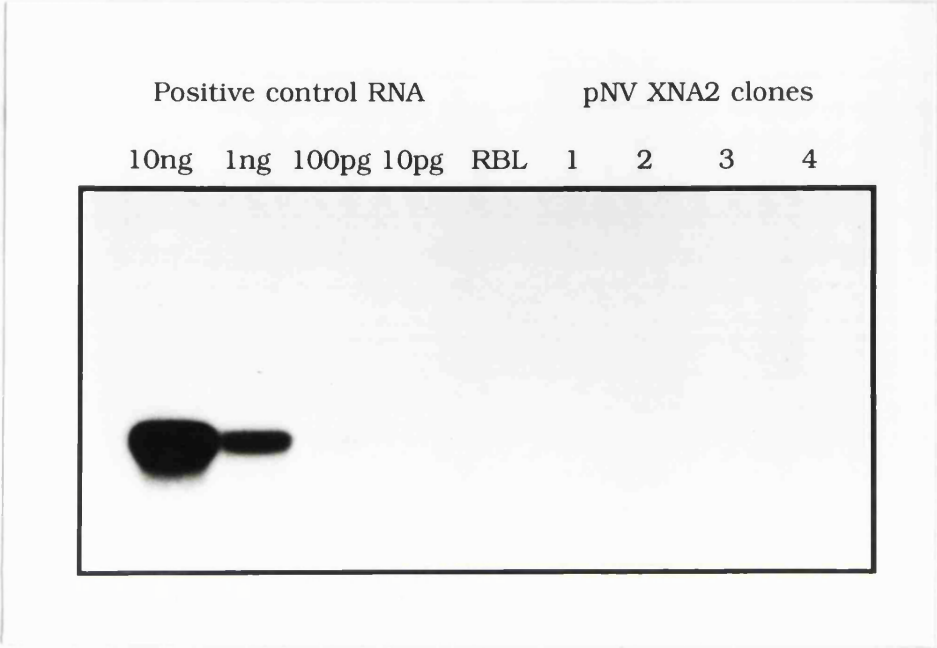


Figure 5.5 *Riboprobe blot of annexin II antisense transcripts in RBL-2H3 cells transfected with pNV-XNA2*
The first four tracks contain the indicated amounts of *in vitro* transcribed positive control RNA. The fifth track contains RNA isolated from parent RBL-2H3 cells. The remaining tracks contain RNA isolated from four pNV-XNA2-transfected clones. The control transcripts are approximately 900 bases; the antisense transcripts in RNA isolated from transfected clones should be 1.5kb.

From this it was concluded that although the single promoter responsible for driving transcription of the neomycin resistance gene and the inserted cDNA was active in RBL-2H3 cells, it did not appear to produce transcripts from the cDNA. This could be because the promoter can act at this distance to promote RNA polymerase binding to the start of the neomycin gene, but the polymerase does not recognise the start of the inserted antisense DNA. Alternatively the promoter may be only weakly active in these cells - adequate for the cells to survive in neomycin but not enough to generate detectable quantities of antisense transcripts. Another possible explanation is that when the plasmid becomes incorporated into the host genomic DNA, it preferentially linearises at a point between the promoter and the start of the insert or in the insert

itself. In this case cells will only survive if the neomycin resistance gene is inserted into a region of genomic DNA that is actively transcribed, close to a promoter that stimulates transcription from it. This would contribute to the low efficiency of stable transfection.

Based on these results, no further transfections were attempted with plasmids constructed in pNV and it was decided to consider remaking the constructs in a different vector, a plasmid called pRc/CMV (described in Materials and Methods). This plasmid has the advantage of separate promoters for the neomycin resistance gene and the inserted DNA.

5.5 Constructs for transfection in pRc/CMV

The pRc/CMV vector contains the strong constitutive promoter region from cytomegalovirus to drive transcription of inserted DNA, and has several unique restriction enzyme sites in its polylinker which facilitate directional cloning. To test the efficiency of the neomycin resistance gene in RBL-2H3 cells, whose transcription is driven by the separate SV-40 virus early promoter, transfections were performed with wild type pRc/CMV by both DEAE-dextran and calcium-phosphate protocols. Stable transfectants were created by the DEAE-dextran method, but with very low efficiency (approximately 1 in 10^7 cells). No stable transfectants were created by the calcium phosphate technique.

To test the cytomegalovirus promoter that drives transcription of the inserted DNA in pRc/CMV, a construct containing the cDNA for human annexin V in the sense orientation, CMV-A5, (a kind gift from Dr. P. Smith) was used to transfect RBL-2H3 cells by electroporation. RBL-2H3 cells express annexin V but the rat protein has a different mobility from the human protein on SDS-PAGE (see section 3.1). 18 stably transfected clones were isolated and analysed by SDS-PAGE, Western blotting and immunoblotting with an anti-annexin V antibody (figure 5.6).

The transfected cells contain 3 immunoreactive bands: the upper and lower bands are the two endogenous forms of annexin V found in RBL-2H3 cells; the stronger central band corresponding to transfected protein, indicated by an arrow on the left hand side of each blot, is absent from the control cells. All of the transfected clones

analysed expressed human annexin V, demonstrating that the cytomegalovirus promoter that drives transcription of the inserted DNA is active in RBL-2H3 cells.

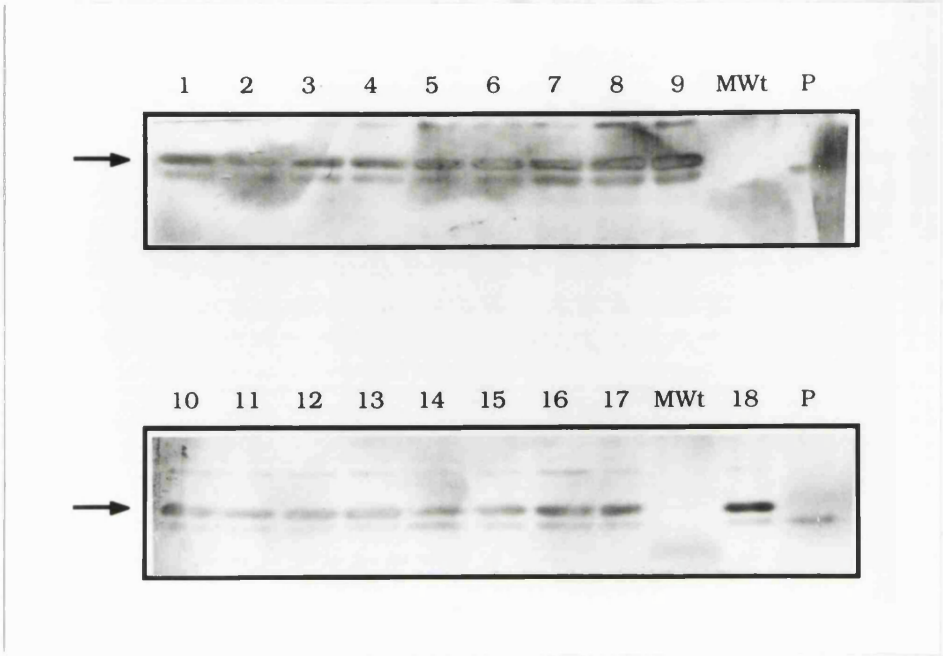


Figure 5.6 *Western blots of RBL-2H3 clones transfected with CMV-A5, immunoblotted for annexin V.*
The right-hand track in each panel, labelled 'P' contains cell lysate from the parent RBL-2H3 line.

5.5.1 Constructs in pRc/CMV containing annexin II cDNA

RNA duplexes are very stable and if they form intramolecularly the secondary structure formation can prevent an RNA strand from hybridising with another oligonucleotide. To reduce the possibility of secondary structure formation occurring in the antisense transcripts, a partial, rather than full, length annexin II cDNA fragment was used to create the antisense construct.

A 350bp fragment of the rat annexin II cDNA, cut from pcDNA1.clone1 with Stu I (nt.350 in the cDNA) and Hind III (in the plasmid polylinker at the 5' end of the cDNA) was isolated from an agarose gel and blunt-end cloned into the Not I-cut vector. Due to the small size of the inserted fragment, its orientation was determined by DNA sequencing using the T7 promoter adjacent to the pRc/CMV polylinker. A plasmid containing the insert in the antisense orientation, CMV-X2, was isolated (figure 5.7 B).

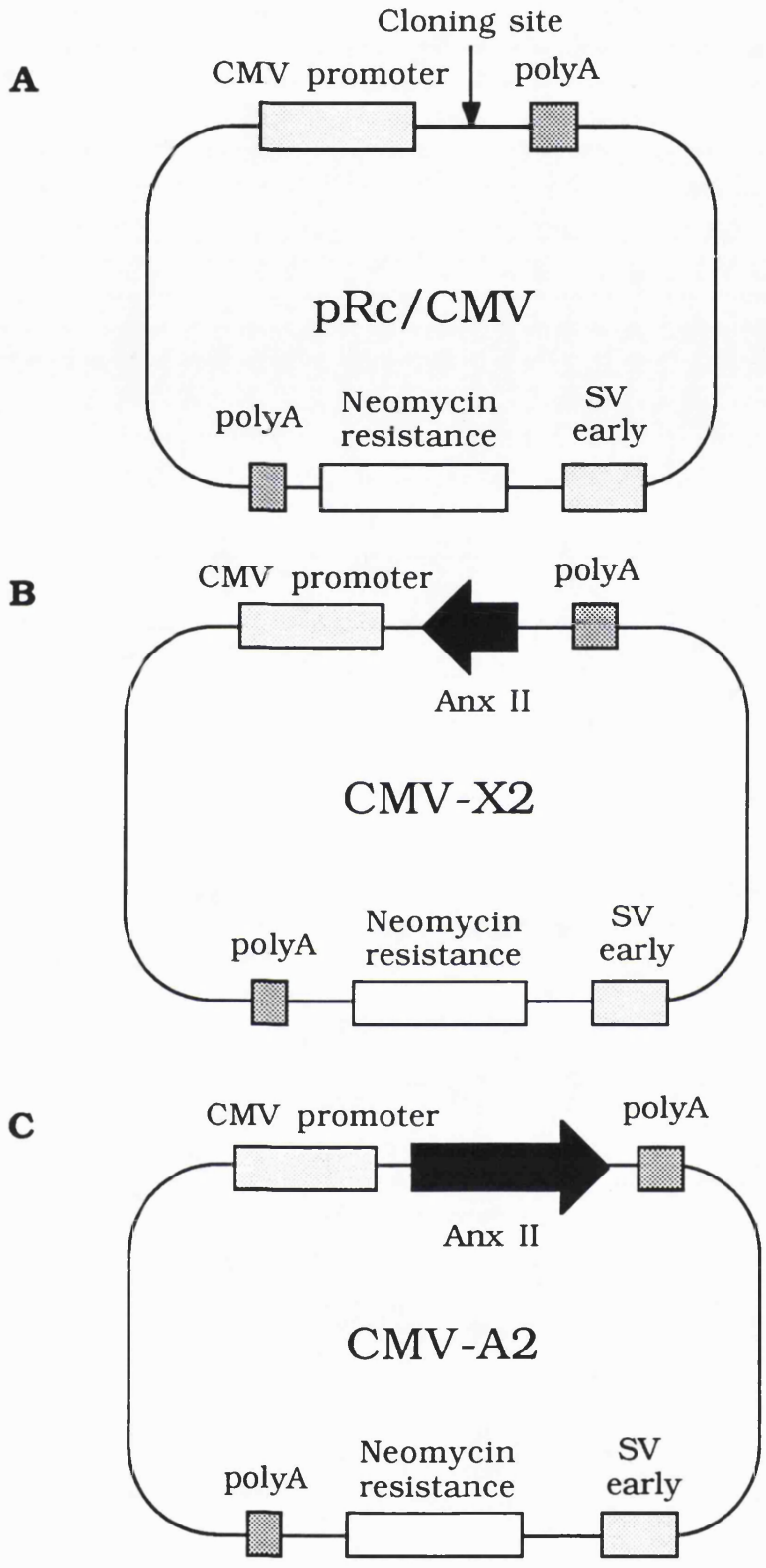


Figure 5.7 *Constructs containing fragments of rat annexin II cDNA in the expression plasmid pRc/CMV*
The viral transcription promoters and polyadenylation signals (dot-filled boxes), and the neomycin resistance gene (empty box) are included. The approximate length and orientation of the annexin II fragments are indicated by the length and orientation of the black arrows.

A fragment of annexin II cDNA containing the entire coding region was cut from pcDNA1.clone1 with Hind III (polylinker) and Xba I (nt.1127), and ligated into Hind III/ Xba I-cut pRc/CMV. This directional cloning produced a construct with the insert in the sense orientation, CMV-A2, to be used for overexpression (figure 5.7 C). The polyadenylation signal, lost from this fragment, is provided in the pRc/CMV plasmid.

5.6 Transfections with CMV-A2 and CMV-X2

Transfection of RBL-2H3 cells with CMV-X2 was performed by electroporation, previously determined to be the most successful method for transfection. Following culture in selection medium, 22 colonies were isolated for analysis. A confluent 30mm dish of each clone was lysed into 250µl of SDS-PAGE lysis buffer. 100µl of each lysate was analysed by SDS-PAGE, Western blotting and immunoblotting with anti-annexin II antibody HH7. The results of the first round of screening are shown in figure 5.8 A and B.

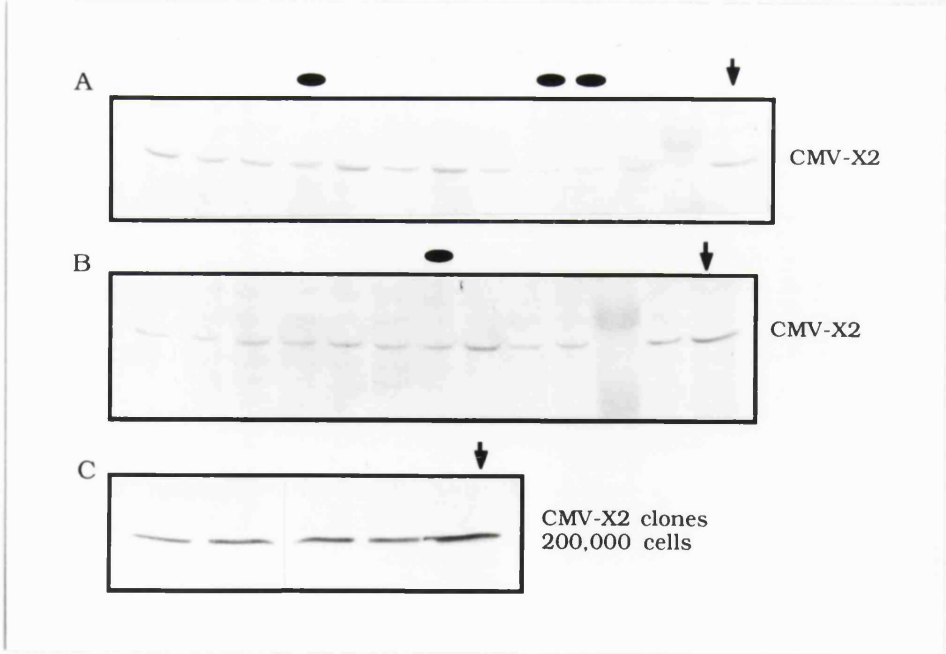


Figure 5.8 *Western blots of clones transfected with CMV-X2, immuno-blotted for annexin II*
The initial screening of 22 colonies for annexin II is shown in panels A and B. Those shown with a black mark above the track were chosen for a second cell-counted gel, the results of which are in panel C. The right hand track in each case, indicated by an arrow, is control RBL-2H3 cells.

Although none of the clones appeared to have significantly reduced their levels of annexin II expression in this initial screening, four clones were chosen for a more accurate comparison of annexin II levels, the rest were discarded. Dishes of these four clones transfected with CMV-X2, were trypsinised and cell counted prior to lysis. Equal cell number loadings (200,000 cells) were analysed as above, the results of immunoblotting for annexin II are shown in figure 5.8 C.

Since none of these clones showed a marked difference in annexin II content, RNA was prepared from 18 of the CMV-X2-transfected clones and from the parent RBL-2H3 cell line to be riboprobed for the presence of antisense transcripts. Radiolabelled annexin II sense strand RNA was prepared by *in vitro* transcription from a construct in pBluescript containing the first 1100bp of rat annexin II (figure 5.9).

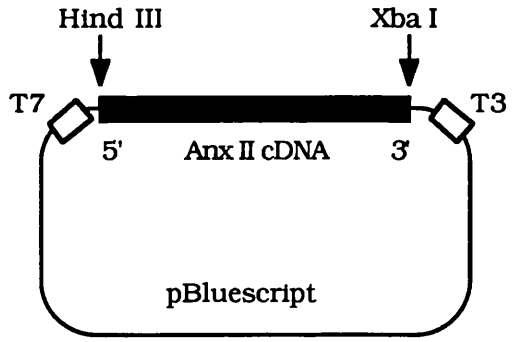


Figure 5.9 *Schematic representation of the template for in vitro transcription of sense and antisense annexin II RNA*
This figure shows the restriction sites used to linearise the plasmid and the position of the T7 and T3 RNA polymerase promoters (empty boxes) either side of the inserted 1100bp fragment of annexin II cDNA (black box).

The plasmid was linearised with Xba I, and RNA transcribed from the 5' end using T3 RNA polymerase. Positive control antisense annexin II RNA was transcribed from the same template linearised with Hind III using T7 RNA polymerase. Positive control RNA was quantified as described in section 5.4.1.

20µg of RNA from each of 18 clones and 2 dishes of transfected cells each containing about 100 separate clones (in case the frequency of expression of the

antisense insert was low) were electrophoresed and Northern blotted along with various loadings of positive control RNA. The Northern blot was probed with the radiolabelled sense strand RNA as described above. The autoradiograph of the riboprobe is shown in figure 5.10.

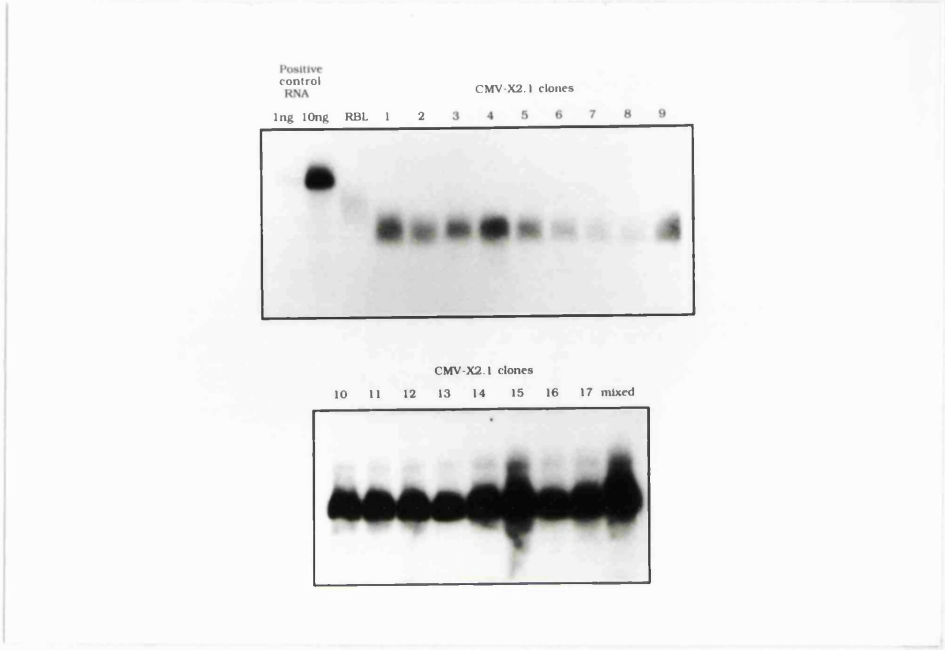


Figure 5.10 *Riboprobe for the presence of annexin II antisense transcripts in RBL-2H3 cells transfected with CMV-X2*
Upper panel: The first two tracks contain in vitro transcribed positive control RNA. The track marked RBL contains RNA isolated from the parent RBL-2H3 cells, the remaining tracks are from CMV-X2-transfected clones. The lower panel shows the riboprobe of another 9 clones after a longer exposure time. The control transcripts are approximately 1.1kb; the antisense transcripts in RNA isolated from transfected clones are 350 bases long.

10ng of control RNA was detected on these blots. Antisense transcripts were detected in all of the CMV-X2-transfected clones tested, so it was decided to perform further transfections and isolate fresh clones.

A further 20 CMV-X2 clones were isolated and analysed as before, along with 34 CMV-A2 transfected clones. The annexin II immunoblots of these clones are shown in figure 5.11. Panels A and B show clones transfected with the antisense vector; panels C, D and E show clones transfected with the sense vector. Clones exhibiting altered levels

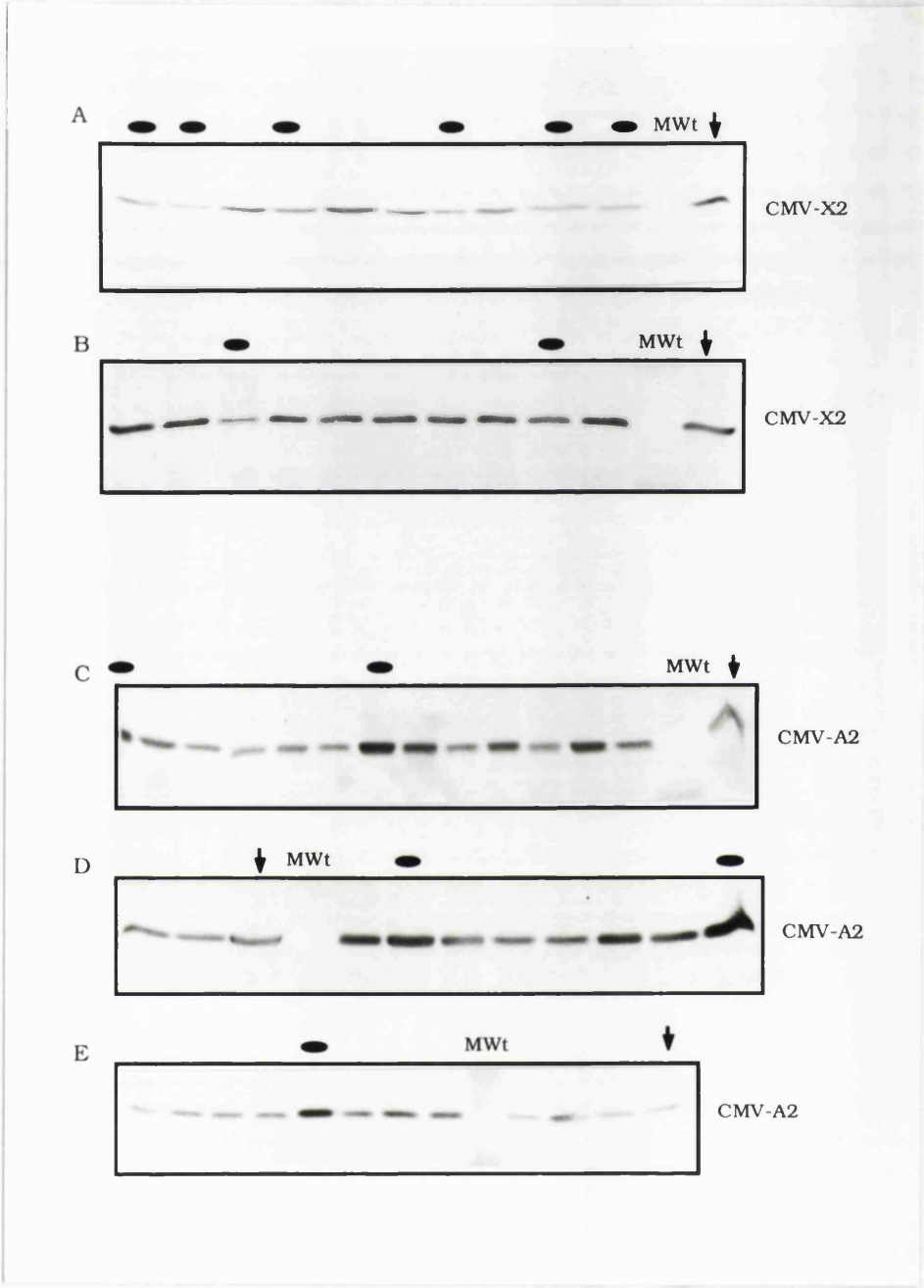


Figure 5.11 *Western blots of clones transfected with CMV-X2 and CMV-A2 immunoblotted for annexin II*
Panels A and B contain clones transfected with CMV-X2; panels C, D and E contain clones transfected with CMV-A2. The track indicated by an arrow in each panel contains control RBL-2H3 cell lysate. Clones chosen for further analysis are indicated by a black mark over the track containing them.

of annexin II, chosen for more accurate analysis are indicated by a black mark above their tracks in figure 5.11.

60,000 cells from each of five CMV-A2-transfected clones (CMV-A2-A+ to -E+) and eight CMV-X2-transfected clones (CMV-X2-A- to H-) were immunoblotted for annexins I and II (figure 5.12 A, upper and lower panels).

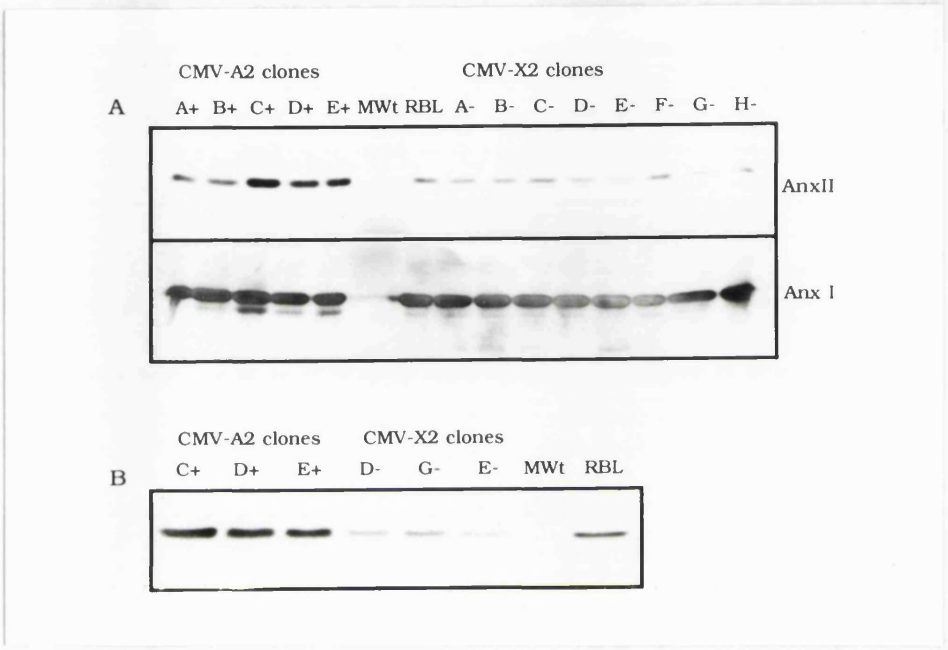


Figure 5.12 *Western blots of clones transfected with CMV-X2 and CMV-A2 immunoblotted for annexins I and II.*
A) upper panel, 60,000 cells immunoblotted for annexin II; A) lower panel, 15,000 cells immunoblotted for annexin I. B) 250,000 cells of each clone indicated, immunoblotted for annexin II. The track labelled "RBL" contains control RBL-2H3 cell lysate. The tracks containing molecular weight markers are labelled "MWt".

The annexin I levels of all clones chosen for further analysis were approximately identical, confirming that the transfected DNA did not interfere with the expression of this very similar protein. All five CMV-A2 clones appeared to overexpress annexin II, in particular CMV-A2-C+, D+ and E+. Only three of the CMV-X2 clones, D-, E- and G-, showed a greater than approximately 50% reduction in annexin II levels, judged by eye. This can be seen more clearly in figure 5.12 B where 250,000 cells from the six clones mentioned are blotted in each track. Clone CMV-X2-E- expressed less than about 25% of normal annexin II levels, and CMV-A2-C+ exhibited an approximate 3 to 4-fold

increase in annexin II levels (judged by eye). These two clones were chosen for analysis of their secretory characteristics (section 5.8). Three weeks later (day 45 after transfection) the annexin II levels of these cells were checked again by immunoblotting (figure 5.13).

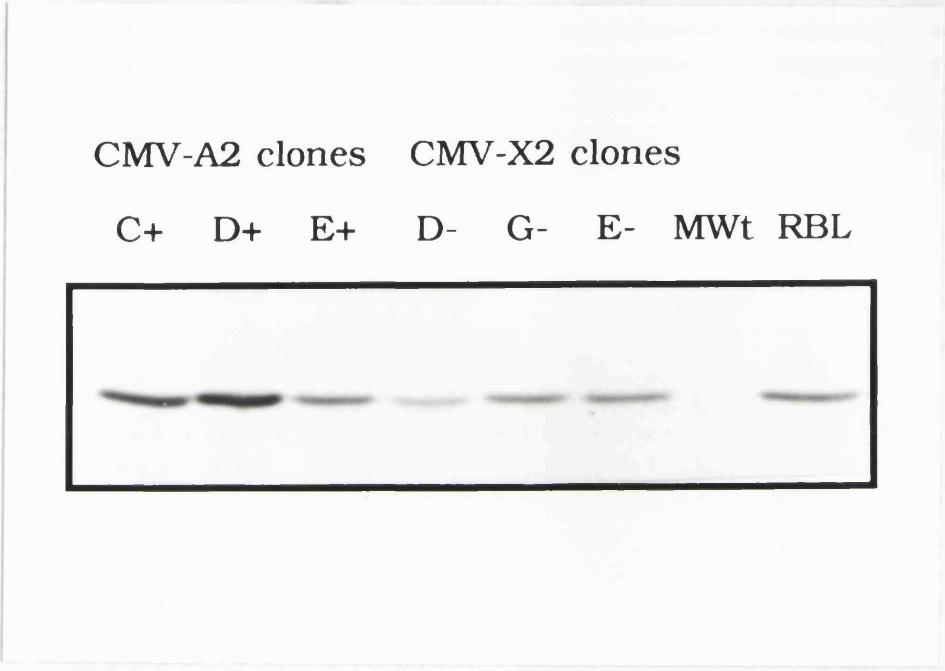


Figure 5.13 *Western blot of clones transfected with CMV-X2 and CMV-A2, immunoblotted for annexin II three weeks after the initial screening.* Each track contains protein from 200,000 cells. The track labelled "RBL" contains control RBL-2H3 cell lysate. The track containing molecular weight markers is labelled "MWt".

The result, using 200,000 cells, shows that the levels of annexin II expressed by the CMV-X2-transfected clones had increased, with only CMV-X2-D- remaining noticeably below normal (approximately 30-50%). Annexin II expression in CMV-A2-transfected cells had decreased, although it remained above normal in clones CMV-A2-C+ and -D+.

5.7 Analysis of fluctuations in annexin II levels of transfected and wild type RBL-2H3 cells

Because Western blots showed expression levels of annexin II in transfected clones were apparently changing, the possibility was investigated that these

fluctuations were related to the growth state of the cells, e.g. degree of confluence. The 'under-expressing' clone CMV X2-E-, and the 'over-expressing' clone CMV-A2-C+, as well as wild-type RBL-3H3 cells were examined.

Four 3cm dishes of each cell type were plated at low density in fresh medium. Each day for the next four days one dish of each cell type was harvested into 250µl of lysis buffer. The cells were at approximately 20% confluence on day one, 40% on day two, 80% on day 3 and confluent with cells dying on day 4. Cells were re-fed at the end of days two and three. The protein content of each cell lysate was determined using the BioRad Protein Assay. 20µg of protein from each cell type was analysed by SDS-PAGE and Western blotting, followed by immunoblotting with the anti-annexin II antibody HH7 and anti-annexin I antibody anti-p35I. The results are shown in figure 5.14, A and B.

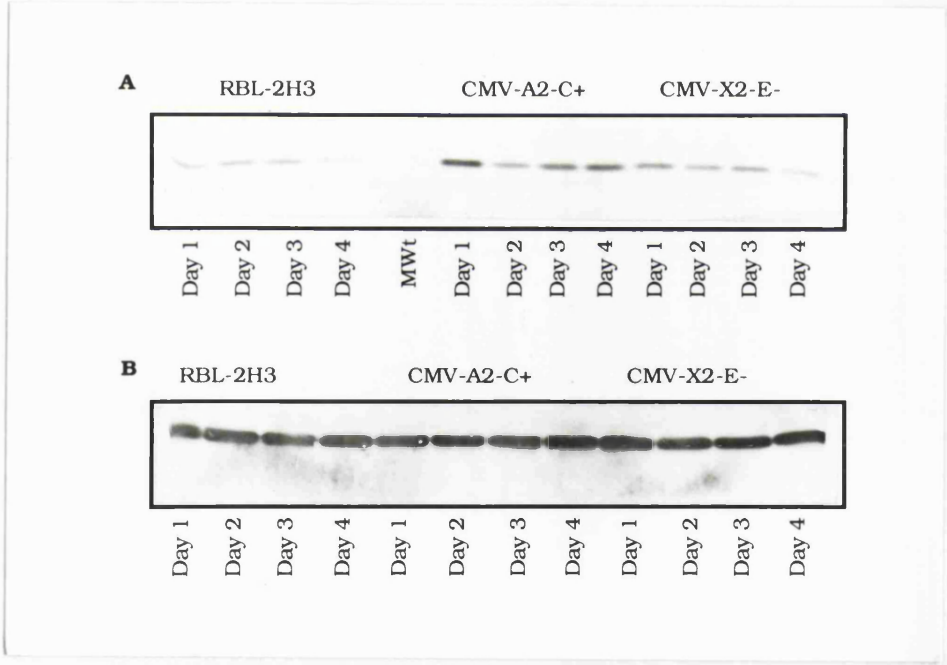


Figure 5.14 *Western blot of cell lysates from control and transfected RBL-2H3 cells at different degrees of confluence immunoblotted for annexins 1 and II.*
Each track contains 20µg of protein from either clone CMV-A2-C+, clone CMV-X2-E- or control RBL-2H3 cells. Panel A is immunoblotted for annexin II; panel B is immunoblotted for annexin I.

Minor variations between annexin II levels in cells harvested on different days occur, with the least confluent cells (Day 1 tracks in figure 5.14 A) expressing more annexin II than confluent cells (Day 4) for all three cell lines. However the difference between the levels of annexin II expressed by the different clones was greatly reduced compared to earlier western blots of these clones. Annexin I levels were similar in all three cell lines. These blots demonstrate that rather than fluctuating, the level of annexin II expression in the "underexpressing" clone CMV-X2-E- had reverted to normal, and annexin II expression had fallen in CMV-A2-C+, although it still displayed slightly increased annexin II expression compared to wild type RBL-2H3 cells.

5.8 Secretory analysis of transfected clones

Clones CMV-X2-E- and CMV-A2-C+ were analysed for their ability to secrete in response to the effectors Ca^{2+} and GTPγS after permeabilisation with SL-O. The results from individual experiments are shown on the following pages (figures 5.15-5.17). These experiments were repeated several times before the clones were immunoblotted for annexin II again, and their annexin II levels were found to have changed .

The secretion assays performed on these clones between days 30 and 36 after transfection revealed that the secretory response to pCa5.25 and a range of GTPγS concentrations was markedly depressed in CMV-X2-E-, the clone that under-expressed annexin II, and was normal or possibly slightly enhanced in CMV-A2-C+, the clone over-expressing annexin II. The maximum response of clone CMV-X2-E- on day 36 (pCa5.25, 10μM GTPγS) appeared to be slightly greater than in the previous assays (approximately 30% as opposed to 22%).

When the assay was repeated on day 45 after transfection (figure 5.17), the secretory responses of the two clones were indistinguishable from wild-type RBL-2H3 cells (not shown). A western blot of the clones performed after 45 days revealed that annexin II expression in both clones had returned to near normal (figure 5.13). Due to the unstable nature of the clones it was not possible to examine the reproducibility of the secretion results obtained within the time constraints of this thesis.

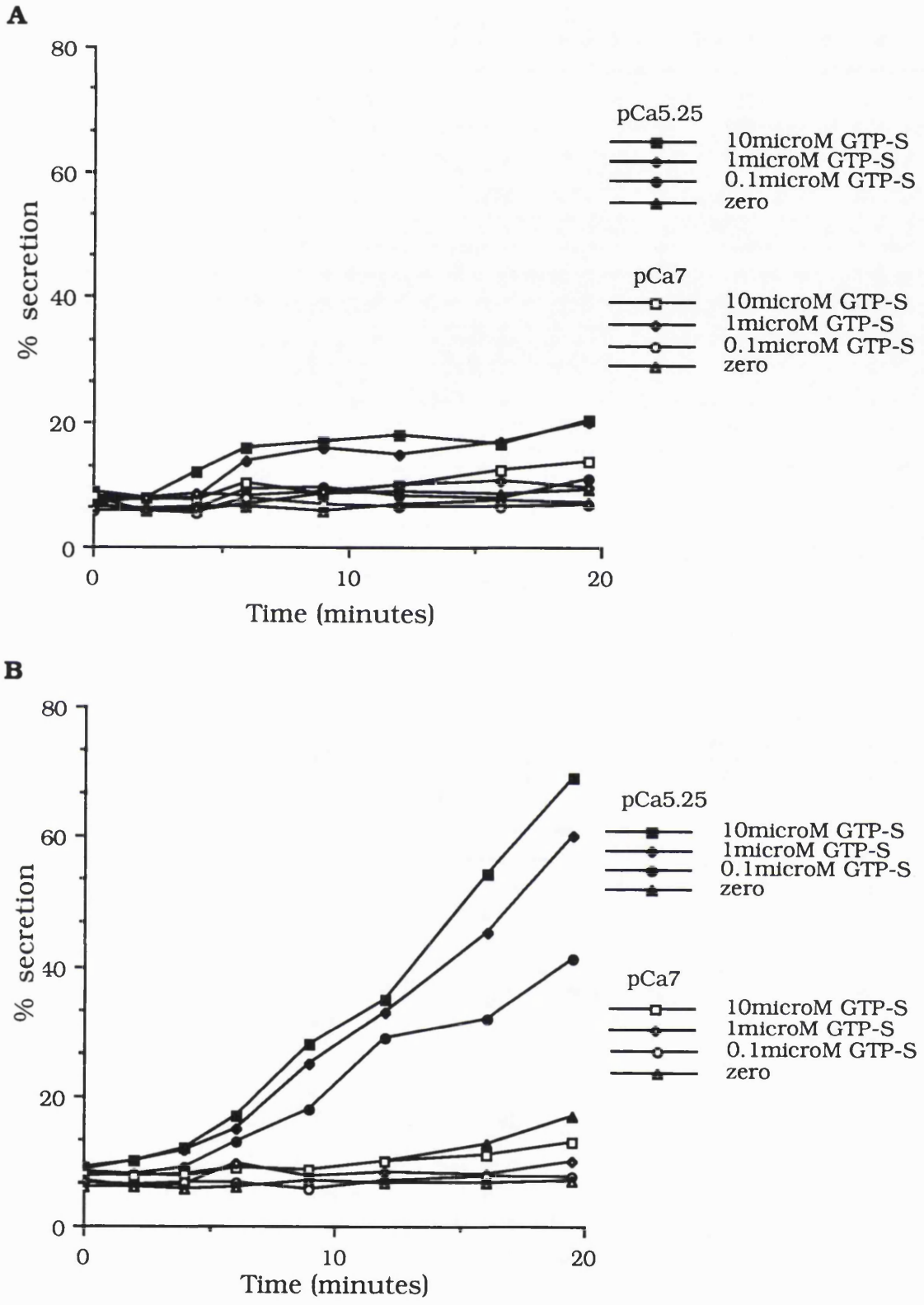


Figure 5.15 *Time course for secretion from CMV-X2-E- and CMV-A2-C+ on day 30 after transfection*
Cells were permeabilised with SL-O and incubated for the indicated times in the presence of pCa 5.25 or pCa7 and μM GTPγS concentrations as shown (microM GTP-S). Secretion was stopped by the addition of EDTA. Each point was done in duplicate. A) Secretion from clone CMV-X2-E-. B) Secretion from clone CMV-A2-C+

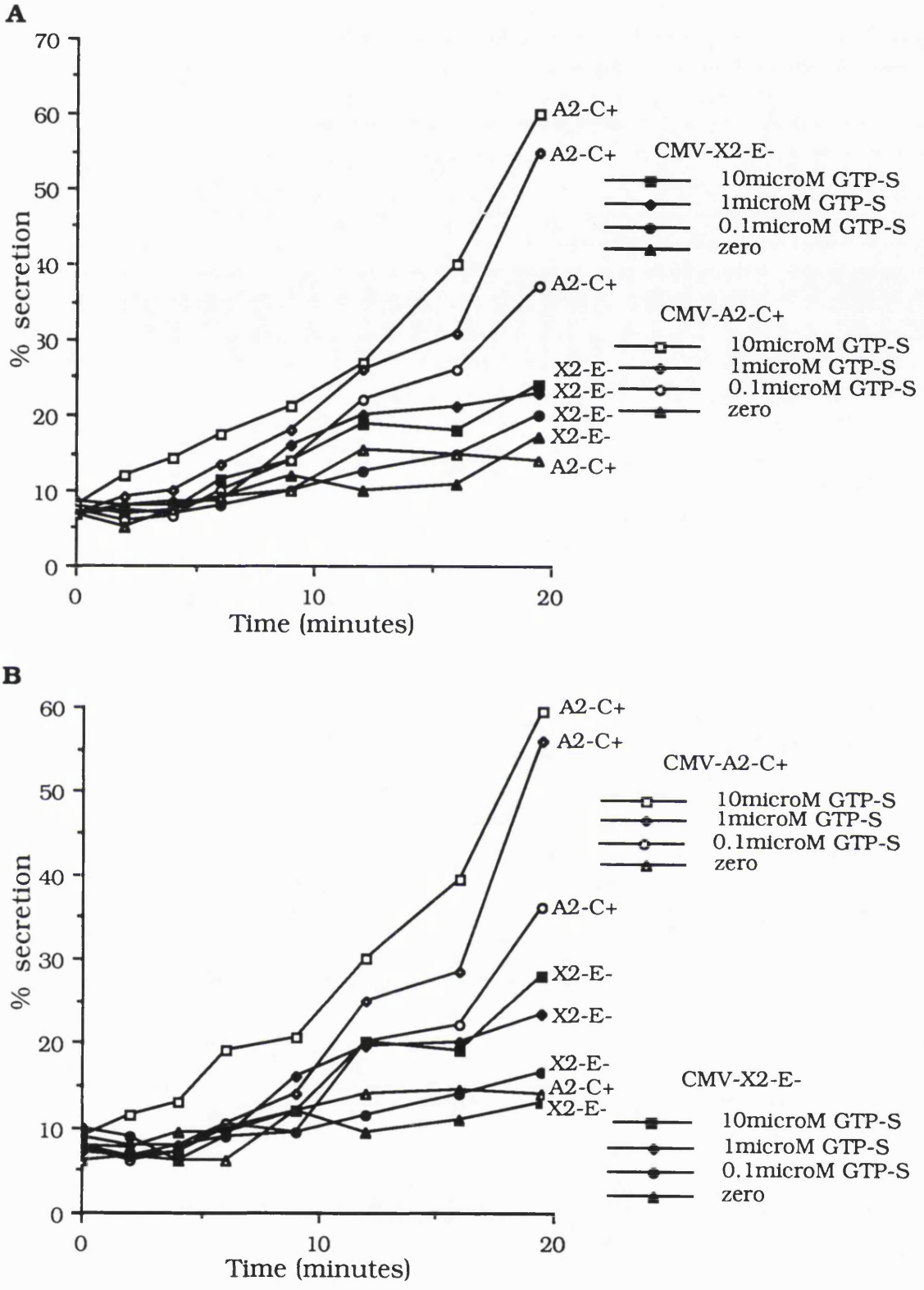


Figure 5.16 *Time course for secretion from CMV-X2-E- and CMV-A2-C+ on day 33 and 36 after transfection*
Cells were permeabilised with SL-O and incubated for the indicated times in the presence of pCa 5.25 and μM GTPγS concentrations as shown (microM GTP-S). Secretion was stopped by the addition of EDTA. Each point was done in duplicate. Lines corresponding to each clone are identified by A2-C+ for CMV-A2-C+, and X2-E- for CMV-X2-E-. A) Secretion assays performed on day 33. B) Secretion assays on day 36.

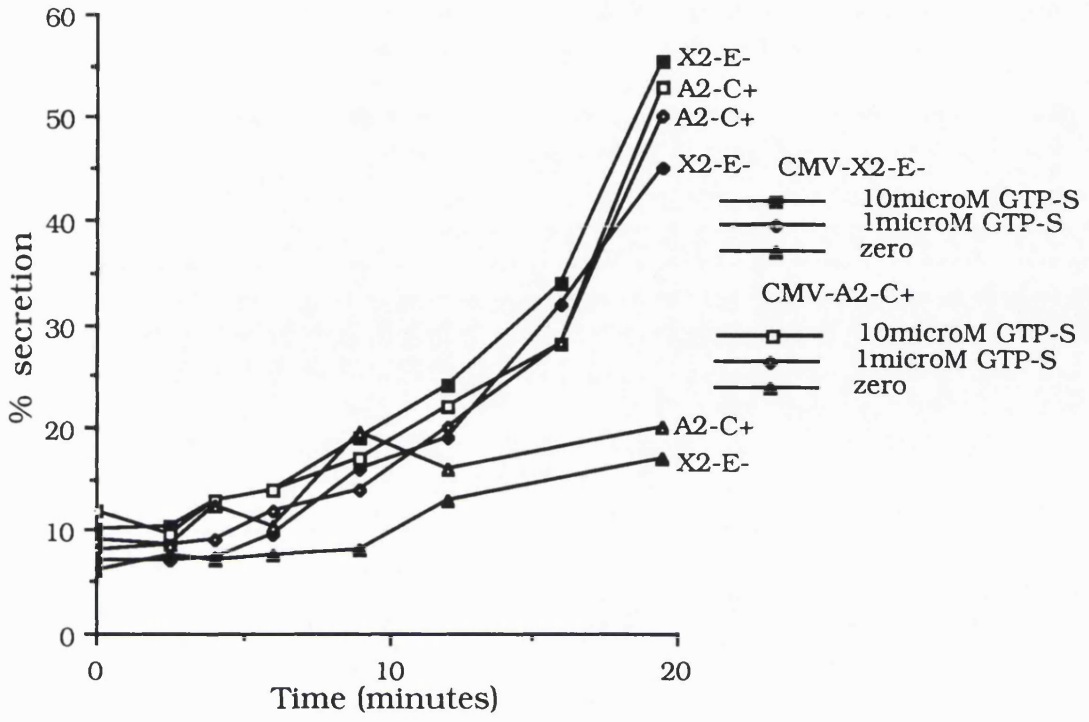


Figure 5.17 *Time course for secretion from CMV-X2-E- and CMV-A2-C+ on day 45 after transfection*
Cells were permeabilised with SL-O and incubated for the indicated times in the presence of pCa 5.25 and μM GTPγS concentrations as shown. Secretion was stopped by the addition of EDTA. Each point was done in duplicate. Lines corresponding to each clone are identified by A2-C+ for CMV-A2-C+, and X2-E- for CMV-X2-E-.

5.9 Antisense oligonucleotides for reducing annexin II expression

An alternative method was used to attempt to transiently reduce the level of annexin II expression in RBL-2H3 cells. Others have reported that short oligonucleotides added to the external medium are internalised by some cell types, and can hybridise with mRNA. Good results have been achieved with this method both in cell culture and recently also in vivo: antisense oligodeoxynucleotides complementary to the NMDA receptor R1 mRNA have been used to reduce the volume of the focal ischaemic infarction produced by occlusion of the middle cerebral artery in the rat (Wahlestedt *et al.*, 1993). It is not known how short oligonucleotides are transported into the cell and it appears to be impossible to predict whether a particular cell type will

be receptive to this treatment, or whether a particular oligonucleotide will interact effectively with the target mRNA.

An oligonucleotide was designed to cover the initiation codon, extending from nucleotide -4 to +15. This was based on the reports of Harel-Bellan *et al.* (1988) who used a 15-mer covering the translation start site of c-*myc*; Sumikawa and Miledi (1988) who used a 19-mer complementary to codons for amino acid residues 4 to 10 of the nicotinic acetyl choline receptor; and Jessus *et al.* (1988) who used a 27-mer complementary to nucleotides 1200-1226 of β-tubulin mRNA.

Rat annexin II cDNA : 5' ..caaaATGTCTACTGTCCAC ..3'

Antisense oligo : 5' GTGGACAGTAGACATTTTG 3'

This was checked by eye for the possibility of secondary structure formation and none was found. It has been reported that phosphorothioate-modified oligonucleotides show enhanced binding affinity for RNA and are more stable since the phosphorothioate linkage is less sensitive to intracellular nucleases, including RNase H. Therefore a phosphorothioate derivative of the required oligonucleotide was obtained. The cells were exposed to the oligonucleotide as described below for up to three days to ensure time for pre-existing annexin II to be turned over. The experiment was conducted at two different cell densities in case cell density had any effect on oligonucleotide uptake.

Two sets of four 3 cm dishes, A-D, of RBL-2H3 cells were plated at densities of approximately 5% and 20% confluence. The following day (day 1) all dishes were given fresh medium, but dish A received fresh medium containing the oligonucleotide at 50μg/ml. On day 2 all dishes received fresh medium, but dishes A and B received medium containing the oligonucleotide. On day 3 all dishes again received fresh medium, and this time dishes A, B and C received medium containing oligonucleotide. On day 4 all four dishes were rinsed in PBS and lysed into 250μl of lysis buffer for analysis of their annexin II levels by SDS-PAGE, Western blotting and immunoblotting for annexin II with HH7. The lower density cells were virtually confluent by day 4,

while the higher density cells reached confluence by day 2. An equal volume of each sample was used. The result is shown in figure 5.18.

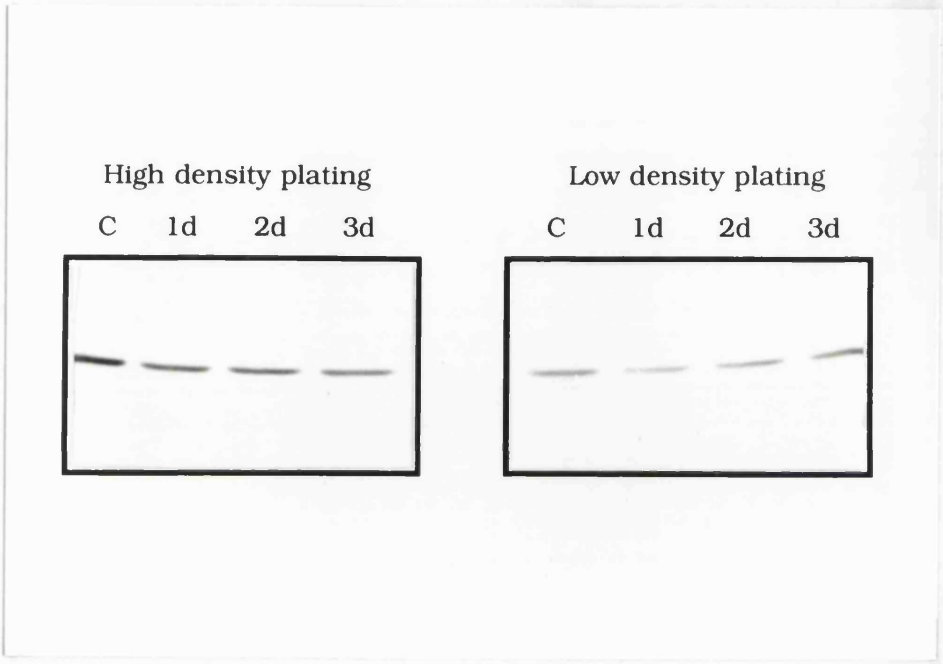


Figure 5.18 *Western blot of RBL-2H3 cells exposed to an oligonucleotide complementary to the translation start site of annexin II mRNA*
The tracks contains cells grown in medium containing the oligonucleotide for no ('C'), 1, 2 or 3 days (1d, 2d and 3d respectively) before harvesting. The relative density of the cells is indicated.

The levels of annexin II were not significantly altered by exposure to the antisense oligonucleotide, and cell density did not appear to influence the result. There are a number of possible reasons for the failure of the antisense oligonucleotide to have an effect, the most likely of which is that the oligo is not taken up by the cell. As mentioned before, the mechanism of transporting short oligonucleotides across the plasma membrane is unknown and not all cell types are capable of performing this function. Alternatively, the oligonucleotide may enter the cells but then be degraded by cellular DNases before coming into contact with its target mRNA.

This approach was not pursued too vigorously as a study with *Xenopus* oocytes found that antisense oligonucleotides probably cause some degradation of many other RNA species as well as their complementary target (Woolf *et al.*, 1992).

5.10 Discussion

5.10.1 Transfection experiments

In an attempt to investigate the role of annexin II in exocytosis from intact RBL-2H3 cells several strategies were employed designed to alter the levels of annexin II expression. Attempts to achieve this with the expression plasmid pNV were unsuccessful, so a second vector was employed: pRc/CMV. The establishment of stable transfectants with pRc/CMV demonstrated that the SV-40 early promoter, that drives transcription of the neomycin resistance gene in this vector, is active in RBL-2H3 cells. Transfections with the construct containing the annexin V cDNA demonstrated that the cytomegalovirus promoter that drives transcription of the inserted DNA is also active in RBL-2H3 cells. The levels of annexin V expressed by these clones, all of which produced strong bands on Western blots, contrast with the limited success of overexpressing annexin II: only 30-40% of clones in figure 5.11 showed overexpression of annexin II and in these it was four-fold increase over normal at best. It is suprising that the annexin II expression vector was less successful than the annexin V expression vector given that all clones must have incorporated the plasmid into their genome to survive in neomycin. The lower success rate with the annexin II expression plasmid suggests that a feedback mechanism exists to down-regulate transcription from the endogenous annexin II gene, to compensate for extra annexin II being produced from the viral promoter-driven cDNA, and prevent large changes in annexin II levels. Alternatively, since annexin II is a fairly abundant cellular protein (0.1-1% of cellular protein), a few-fold increase in production might correspond to a rather large amount of protein synthesis. The anti-annexin V antibody may be considerably more sensitive than the anti-annexin II antibody, in which case a small amount of annexin V produces a strong band whereas the increase in annexin II expression which may represent a substantial increase in protein synthesis, is only seen as a small percentage increase in annexin II. Without knowing the relative affinities of the antibodies for annexins II and V, it is not possible to quantitatively compare the amounts of protein produced.

There are several possible reasons for the low success rate of reducing annexin II levels. Riboprobes of RNA from transfected cells confirmed that antisense annexin II

RNA was being transcribed which means that either secondary structure formation by the antisense RNA prevents it interacting with mRNA, or, antisense transcripts produced combine with endogenous mRNA but not in sufficient quantities to affect protein levels. It is also possible that these cells have a feedback mechanism to upregulate transcription from the endogenous gene to compensate.

The fact that the under-expressing clones reverted to normal levels of expression suggests that there is a strong selective pressure to produce normal quantities of annexin II. Perhaps cells able to increase production of endogenous message, or that lose the antisense insert, have a growth or survival advantage over those that cannot. Alternatively the insertion may simply have been unstable and spontaneously rejected over a period of weeks.

5.10.2 Secretion experiments

The secretion studies performed on clone CMV-X2-E- 30 to 36 days after transfection demonstrated that its secretory response to Ca^{2+} and GTPγS were reduced when compared to wild type RBL-2H3 cells. This was observed over a period of 6 days, but 9 days later the secretory response was found to have returned to a level similar to that of the parent RBL-2H3 cell population. The reversion to wild-type levels of secretion was found to be associated with a return to wild-type levels of annexin II expression. Clone CMV-A2-C+ showed no significant alteration in secretory ability from wild type RBL-2H3 cells. This indicates that the amount of annexin II is not a rate-limiting factor in secretion from RBL-2H3 cells.

Although these results are potentially very interesting and support a role for annexin II in secretion, the instability of the clones prevented in depth analysis of secretory characteristics, and further experiments are necessary to link reduction in secretory capability unequivocally to the loss of annexin II from these cells.

Chapter 6

Discussion

6.1 Comparison of known annexin II protein sequences

An intrinsic part of the investigation into the role of annexin II in secretion from a rat cell line was the cloning and sequencing of a rat annexin II cDNA (Upton and Moss, 1994). Figure 6.1 shows the amino acid sequence of rat annexin II aligned with those of the mouse, cow, human, chicken and *Xenopus* homologues. Comparisons of the avian, amphibian and mammalian annexin II amino acid sequences reveal this annexin to be highly conserved among chordate phyla. Avian and mammalian annexin II are 90% identical to each other, while *Xenopus* annexin II is 80% identical to both the mammalian and avian proteins. The protein sequence identity between rat and murine, and rat and human annexin II is 97%. Murine and human annexin II show a similar high degree of sequence similarity to each other (98%). The two *Xenopus* isoforms of annexin II share slightly less identity with each other (93%) than annexins II from different mammalian species, suggesting that the appearance of two forms in *Xenopus* occurred before speciation of the mammals.

The protein sequence deduced from the rat annexin II cDNA sequence contains 9 amino acid substitutions compared to the murine protein, and 11 compared to the human protein. All but four of these substitutions are conservative, swapping one non-polar residue for another, acidic for acidic, or basic for basic. Only the proline-28 (in rat and mouse) for alanine (human), proline-83 (in rat and mouse) for alanine (in human), cysteine-225 (rat and mouse) for proline (human) and the phenylalanine-320 (rat) for tyrosine (mouse and human) substitutions are non-conservative. Proline imposes constraints on backbone shape that prevent α-helix formation. Residue 28 lies at the end of the N-terminal tail adjacent to the core region for which α-helical structure is not proposed. Proline-83 is at the start of the fourth α-helix (*d*) in the first repeat (based on the structure of annexin V determined by Huber *et al.* (1990a)), the acidic residue

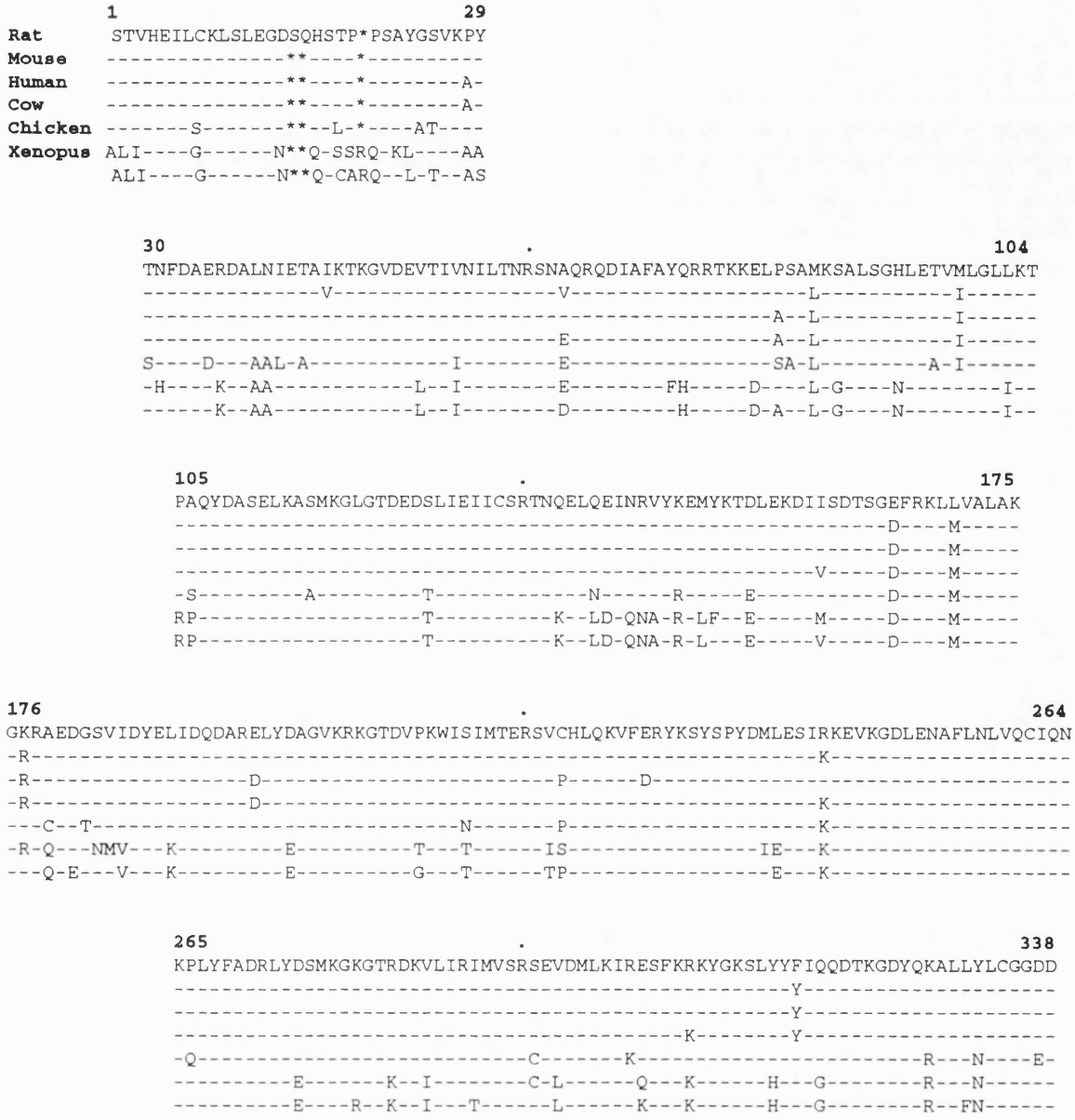


Figure 6.1 *Alignment of the known annexin II protein sequences*
The linear sequences are arranged to reflect the domain structure of the protein: the unique N-terminal domain is followed by the four repeated domains of the C-terminal core. The repeated domains are aligned with each other according to the position of an invariant arginine residue, indicated by a dot above each repeat. Both known isoforms of *Xenopus* annexin II are included. Identical amino acids are represented by bars. Stars indicate the position of the residues inserted into rat annexin II and *Xenopus* annexin II. Sequence information is taken from Gerke *et al.* (1991), Saris *et al.* (1986), Kristensen *et al.* (1986) and Spano *et al.* (1990).

located at the end of this helix in other repeats as part of the consensus calcium-binding site is lost from this repeat (see figure 1.1), i.e. the calcium binding site is non-functional and so the interruption of this α-helix should not affect calcium-binding. Residue 225 is at the boundary of helices *b* and *c* in the third repeat, thus the introduction of a proline residue will not affect helix formation and is not a significant change in the size of the side chain from cysteine. The substitution of phenylalanine-320, in helix *d* of the fourth repeat, for the physically similar tyrosine is not likely to alter protein folding, but may indicate that this residue is located on the surface of the molecule where changes in charge distribution are less likely to interfere with other residues.

The only significant difference between the rat annexin II described here and those from other species (as well as the rat annexin II sequence published by Ozaki and Sakiyama, 1993) is the predicted two amino acid insertion between residues 15 and 16, encoded by 6 extra nucleotides at a position corresponding to an intron/exon boundary in the human and murine genes.

6.2 Possible explanations of the rat annexin II hexanucleotide insert

6.2.1 Multiple annexin genes

The N-terminal variation observed between the two rat annexin II cDNA clones has several possible explanations. The first is that the two rat annexin II cDNAs are the products of two separate genes or different alleles at the same locus. Several examples of multiple annexin genes have been described. The pigeon *Columba livia* has evolved a unique annexin I isoform, cp35, expressed only in cropsac epithelium (Hitti and Horseman, 1991). It is the product of a distinct gene from the other annexin I homologue, cp37 (Haigler *et al.*, 1992). The two annexin I genes are 91% identical and presumably arose through a chromosomal aberration which resulted in two copies of the gene being generated. cp35 is unrelated to mammalian annexin I in the region of the N-terminus containing the phosphorylation sites (the "hinge region" connecting the N-terminus to the core) but is subject to transcriptional control by prolactin. cp37 also

contains little identity to mammalian annexin I in the hinge region, however it appears to have independently evolved sites here for EGF and PKC phosphorylation (Horseman, 1992), suggesting that control by phosphorylation is important.

Learmonth *et al.* (1992) have isolated two forms of annexin V from mammalian brain. They are identical apart from two amino acid substitutions, but migrate on SDS-PAGE with an apparent 4kD difference, despite only a 15Da difference in calculated molecular weights. These two forms could represent allelic differences or be the products of two separate genes.

Xenopus laevis has two separate functional annexin II genes (Gerke *et al.*, 1991). These encode two 93% identical proteins with 24 amino acid substitutions distributed along their entire length. There are also multiple annexin II genes in the human genome, but only one is functional, the other three are pseudogenes (Spano *et al.*, 1990). The pseudogenes contain no introns but have several deletions and/or substitutions, and one contains an internal stop codon. This is in contrast to the murine genome which contains a single annexin II gene locus (Saris *et al.*, 1986). The annexin II gene consists of 13 exons and is at least 40kb in length. The coding sequence starts in the second exon, which contains the first 15 amino acid residues of the protein including the entire p11 binding site (Amiguet *et al.*, 1990). It is at the boundary between this first coding exon (exon 2) and the next, that the 6 extra nucleotides are found in rat annexin II cDNA.

6.2.2 Alternative splicing of a cassette exon

A second possibility is that the 6 nucleotides could represent a separate cassette exon that is alternatively spliced. The phenomenon of alternative splicing has been recorded for several other members of the annexin family: annexin VI has an alternative splice site close to the start of the seventh repeat (Moss and Crumpton, 1990), which produces two proteins differing in size by 6 amino acids. The annexin VI isoforms migrate as a doublet on SDS-PAGE gels. Annexin VII has an alternatively spliced exon of 66 base pairs which produces two forms of the protein differing at their N-termini by 22 amino acids. In contrast to the alternative splicing of annexin VI, alternative splicing

of annexin VII is tissue specific: the larger protein, annexin VIIb, which contains the extra 22 amino acids is expressed mainly in muscle, annexin VIIa is expressed mainly in lung (Magendzo *et al.*, 1991). Annexin XI is also alternatively spliced to include one or other of two exons that encode 37 or 39 amino acids close to the N-terminus (Towle *et al.*, 1992). A 33kDa and a 36kDa form of annexin III have been detected in neutrophils and monocytes respectively from the same species, these may represent alternative splice forms although it is not certain (Le Cabec *et al.*, 1992).

The two *Xenopus* oocyte annexin IIs are approximately 80% homologous to mammalian and avian annexin II over the whole protein, but the region encoded by the third exon of the mammalian gene shares only 65% similarity (Izant and Bryson, 1991). This may represent an alternatively spliced N-terminal exon in *Xenopus*, perhaps one that is specific to the oocyte. However, the presence of a more conventional annexin II homologue has not been established.

6.2.3 Retained splice acceptor or donor sites

Unlike the two forms of *Xenopus* annexin II the two rat annexin II cDNAs are 100% identical apart from the 6 extra nucleotides, it is therefore extremely unlikely that they arise from separate genes. As there is only one other recorded instance of an alternatively spliced exon as small as 6 nucleotides, in the cardiac troponin T gene (Breitbart *et al.*, 1987), the most likely explanation for the observed 6 nucleotide insert is that it represents a retained splice acceptor or donor site.

To examine this possibility, the sequences at either end of the known murine and human annexin II introns were compared with the 6 nucleotides in the rat cDNA (Table 6.1).

The 6 nucleotides inserted into the coding region of the rat cDNA, TCTCAG, conform to the 3' splice acceptor site consensus sequence shown in Table 6.1. Compared to the human genomic sequence there is only one base change between these 6 nucleotides and the 3' splice acceptor sequence (in bold in figure 6.2), and two changes compared to the murine sequence (also in bold).

Table 6.1 *The 6 nucleotides at the 3' and 5' ends of annexin II gene introns*

This table shows the 6 nucleotides of intronic sequence immediately adjacent to the exonic sequence for all known murine and human intron/exon boundaries. This information is from Amiguet *et al.* (1990) and Spano *et al.* (1990). The underlined sequences are from the second intron and are therefore candidate retained sites in the rat cDNA.

5' splice signal		**3' splice signal**	
Human	Murine	Human	Murine
GTAAGC		TTCCAG	
GTAAGT	GTAAGT	CCTCAG	TTGCAG
GTAGGT	GTAGGC	CCATAG	CCACAG
GTACAG	GTACTG	TTGAAG	TTACAG
GTAAAT	GTAAAC	TCTCAG	TCCCAG
GTGAGT	GTGAGT	TCCAAG	CCTCAG
GTTGGT	GTTGGT	CCATAG	CTACAG
GTAAGT	GTATGT	TCGTAG	CCACAG
GTGGGC	GTGGGT	CCTCAG	TCTTAG
GTAAGT	GTGAGC	CCACAG	CCACAG
GTAAGT	GTAAGG	TTTCAG	TTTCAG
GTAAGC	GTAAAG	TTGTAG	CTGCAG

Consensus : $G\ T\ ^{A}/_{G}\ ^{A}/_{G}\ G\ T$ $\qquad$ $^{T}/_{C}\ ^{T}/_{C}\ X\ ^{C}/_{T}\ A\ G$

	EXON 2	INTRON 2	EXON 3
Human	AGC TTG GAG GGT GAT	gtaagt.....**cctcag**	CAC TGT ACA CCC CCA
Mouse	AGC CTG GAG GGT GAT	gtaagt.....**ttgcag**	CAT TCT ACA CCC CCA
Rat	AGC TTG GAG GGT GAT	******.....******	TCTCAG CAT TCT ACA CCC CCA

Figure 6.2 *Intron-exon boundaries at the point of insertion of 6 nucleotides in the annexin II cDNA in rat*
Exonic sequence is in uppercase letters, intronic sequence is in lower case letters, unknown intronic sequence is represented by stars. The hexanucleotide insert in the rat cDNA is shown aligned with the splice signal to which it bears most similarity.

If a normal 3' splice acceptor site is retained in the larger rat annexin II cDNA then sequence just upstream must be acting as an alternative splice acceptor signal. The sequence at this intron-exon boundary in the human gene is as follows:

.....atc**ttttaa**cctcag CAC TGT.....

Bases corresponding to those in bold and underlined would thus form the new splice acceptor signal sequence in the rat gene. It requires only a single base change from the human sequence in this 6 nucleotide domain for them to conform to the 3' splice acceptor signal sequence: ttttaa to ttttag.

The most plausible explanation for the extra 6 nucleotides in the mRNA of rat annexin II is therefore a retained 3' splice acceptor site. However, confirmation of this theory will require structural characterisation of the rat annexin II gene.

6.3 Annexin II N-terminal variation

The N-termini of those annexin II proteins sequenced to date, are compared in figure 6.3 below. Note that there is strong sequence conservation between the mammalian species but considerable variation within other vertebrate phyla. Furthermore the degree of sequence conservation is greater in exon 2 than in exon 3, which implies that the p11 binding site has been subject to more rigid functional constraints during evolution.

```
                    Exon 2    -> | <-              Exon 3              -> |

Rat         MSTVHEILCKLSLEGDSQHSTPP*SAYGSVKPYTNFDAERDALNIETAIKTK

Mouse       ----------------**-----*-----------------------V---

Human       ----------------**-----*-------A-----------------

Cow         ----------------**-----*-------A-----------------

Chicken     --------S-------**--L--*---AT--A-S----D---AAL-A-----

Xenopus1    -ALI----G------N**Q-CAR--L-T--AS------K--AA--------

Xenopus2    -ALI----G------N**Q-SSRQ-KL----AA-H----K--AA--------
```

Figure 6.3 *Alignment of the amino acid residues encoded by exons 2 and 3 of vertebrate annexin II.*
Stars denote a gap where residues are inserted in the protein sequence of another species. Numbering in the text below refers to the human sequence.

The N-terminus is an important regulatory region of annexin II. It contains the known *in vivo* phosphorylation sites for protein kinase C and $pp60^{v\text{-}src}$ (serine-25 and tyrosine-23 respectively) and also the p11-binding domain. It is tempting to speculate that the effect of inserting two amino acids between residues 15 and 16 could affect either the availability of serine-25 and tyrosine-23 to protein kinases, and/or the orientation of the p11 binding domain. Moreover the inserted serine residue itself could be a target for protein kinases although it does not lie within a known kinase consensus sequence (apart from that of glycogen synthase kinase-3 if threonine-18 was already phosphorylated), so this is unlikely. Given the importance of the N-termini of annexins in determining aspects of their function it is possible that the different isoforms of annexin II have related but different cellular functions. This may also be true of the N-terminal splice variants of annexins VII and XI, particularly as the calcyclin-binding domain of annexin XI has been localised to a region including one of the alternatively spliced exons (Tokumitsu *et al.*, 1993).

The two *Xenopus laevis* annexin II isoforms share less sequence identity with one another than do annexin IIs from different mammalian species. Of the residues substituted in the region encoded by exon 2, only one is involved in p11 binding - a conservative substitution of isoleucine for valine - which suggests that p11 binding

remains important to both. Having two copies of the same gene may alleviate selective pressure on one of the copies to maintain its functional integrity, but the divergence of both forms from the mammalian and avian proteins in the region encoded by exon 3 suggests that the sequence constraints on this area are low. Phosphorylation or p11 binding to the N-terminus affects the calcium and phospholipid-binding properties of the core domain (Powell and Glenney, 1987), this communication between the N-terminal domain and the rest of the protein appears not to depend heavily on the intervening residues in the linear sequence. The two forms of annexin II in *Xenopus* both contain an inserted glutamine residue at nearby position 21 which could influence communication between the two domains, just as the two amino acids inserted in the rat protein could influence this interaction. The substitution of tyrosine-23, the conserved $pp60^{src}$ phosphorylation site in annexin II, for leucine in the *Xenopus* protein indicates that tyrosine phosphorylation is not important for annexin II function in this species. However it should be noted that it has been suggested that there is an alternatively spliced exon 3 in *Xenopus* which shows greater identity with the mammalian and avian versions, so the isoforms in figure 6.3 above may not be the best homologues for comparison (Gerke *et al.*, 1991).

Finally, the N-terminal insertion reported here may account for the poor cross-reactivity of rat annexin II with one of the antibodies raised against the N-terminus. αCal 1-15 (antibody 1) appeared to recognise annexin II in RBL-2H3 cells with lower affinity than some background bands (figure 3.3). It is possible that the N-terminal insertion disturbs the epitope recognised by αCal 1-15 preventing recognition of the proportion of the annexin II molecules that contain it (assuming that RBL-2H3 cells coexpress both forms).

Once the existence of both splice forms of rat annexin II has been established at the protein level, their phospholipid-binding, p11-binding, Ca^{2+}-binding, phosphorylation and vesicle aggregation properties could be investigated to characterise differences attributable to the two amino acid insert. Differential expression of the two forms, particularly if functional differences are found, could be investigated by RNase protection assays or primer extension analysis.

6.4 Annexin II and exocytosis

The work in this thesis demonstrates that the use of vectors carrying sense or antisense cDNA, stably transfected into RBL-2H3 cells, can selectively alter annexin II expression, without altering that of the closely related protein annexin I. Experiments performed on the transfected clones within a few weeks of transfection, demonstrated that secretion in response to Ca^{2+} and GTPγS was reduced in RBL-2H3 cells that under-expressed annexin II. It is also interesting that the transfected clones were unstable and that reversion to wild-type levels of annexin II expression coincided with recovery of the secretory response, supporting the hypothesis that annexin II has a role in secretion. I had hoped to demonstrate that loss of annexin II was responsible for the observed decrease in secretory capability in cells under-expressing annexin II by permeabilising and stimulating cells in the presence of purified annexin II, in the expectation that this would restore secretion to normal levels. Clonal variation between RBL-2H3 lines has been documented (Woldemussie *et al.*, 1987) and secretory ability can change up or down in a clonal population in continuous culture (Bingham *et al.*, 1994). However since the magnitude of drift reported within clonal populations is smaller and occurs over a greater time scale than that observed here, spontaneous clonal variation is unlikely to be the explanation for the reduced secretory response of clone CMV-X2-E- cells. Experiments demonstrating that annexin II can reconstitute secretion in under-expressing cells, would confirm this. Reconstitution experiments would also address another possible criticism of this technique: that the antisense RNA may interfere non-specifically with RNA species other than its target and therefore reduce expression of other proteins (a phenomenon that has been documented by Woolf *et al.* (1992) for antisense oligonucleotides in *Xenopus* oocytes).

The first analysis of annexin II levels was performed on isolated clones approximately 3 weeks after transfection. The maximum deviations in annexin II levels from normal were observed in both under- and over-expressing clones at this point; subsequent analysis showed that annexin II levels were converging on normal. There are several possible explanations for this adaptation: increased transcription from the endogenous gene; decreased transcription from the inserted antisense cDNA; or loss of

the inserted DNA (although the neomycin resistance gene must remain). If this reversion to wild type starts immediately after transfection then the changes in annexin II levels must have been even greater initially. Ideally therefore, experiments should be performed on cells as soon as possible after transfection. Subsequent attempts at reducing annexin II levels could employ transient transfection, followed within a few days by analysis of secretory ability. Co-transfection with a plasmid carrying growth hormone cDNA, enables secretion from transfected cells to be monitored in isolation from unaffected cells (Holz *et al.*, 1994). An alternative approach would be to use a vector with an inducible promoter. Use of such a vector has several advantages: stably-transfected clones can be induced to express antisense cDNA on demand, producing a reproducible reduction in annexin II expression; when growing in culture in the absence of the inducer there will be no selective pressure on clones to lose the insert or alter transcription from the endogenous annexin II gene; the secretory ability of the clone can be analysed before and after altering the level of annexin II, allowing clonal variation to be ruled out as a possible explanation of secretory behaviour.

If annexin II is involved in secretion what role might it have? Annexin II is localised to the sub-plasma membrane region in RBL-2H3 cells (figure 3.6 B and C), where it is probably attached to the cytoskeleton. The effect of cytoskeleton attachment on the membrane binding ability of the annexin II tetramer is not known, but assuming that the membrane binding sites are still available, the tetramer could attach secretory vesicles to the sub-plasma membrane cytoskeleton prior to fusion. In RBL-2H3 cells this could only occur after stimulation, when the vesicles move to the cortical region. Alternatively, annexin II may "dock" vesicles at the plasma membrane when intracellular calcium rises, immediately prior to exocytosis, or be involved in compound exocytosis, helping to fuse vesicles to each other.

Electron micrographs of mast cells undergoing exocytosis show the formation of "dimples" in the plasma membrane as the surface invaginates before fusing with granules lying approximately 100nm below the surface (Chandler and Heuser, 1980; Monck and Fernandez, 1994 for a review). It has been proposed that a "fusion scaffold" of proteins is responsible for distorting the plasma membrane in this way. To span the

distance from granule to plasma membrane would require 10-15 molecules the size of annexin II. Annexin II oligomers of this size are unlikely, but filaments of annexin II attached to a cytoskeleton backbone, would provide many membrane binding sites along their length which could sequentially bind to the plasma membrane, drawing membrane into the dimple structure. An alternative scenario is that the tetramer localises to the bottom of the dimple where it could establish contact between plasma membrane and vesicle membrane. Purified annexin II tetramer forms short filamentous structures between aggregated liposomes *in vitro*, and it has been immunolocalised to sites of vesicle attachment to the plasma membrane in stimulated chromaffin cells, where similar filamentous structures can be seen bridging the 10nm gap between granules and plasma membrane (Nakata *et al.*, 1990).

Annexin II binds phospholipid vesicles *in vitro* at calcium levels found in resting cells. Annexin II has been identified as a chromaffin granule binding protein (Creutz *et al.*, 1987). The punctate cytoplasmic bodies immunostained for annexin II in RBL-2H3 cells (figure 3.6 B and C) could be secretory vesicles, known to be located in the perinuclear region (De Matteis *et al.*, 1991). If this is the case annexin II may be bound to vesicles in anticipation of their transportation to the cell surface where it will participate in docking or fusing vesicles to the plasma membrane. Alternatively as annexin II has been shown to bind actin (albeit at mM Ca^{2+} *in vitro* (Glenney, 1986)) it may attach vesicles to the cytoskeleton to facilitate their transport to the plasma membrane when the cell is stimulated to secrete. The reported ability of annexin II tetramer to cause actin bundling (Ikebuchi and Waisman, 1990) suggests a possible causal role in cytoskeleton movements of vesicles: annexin II could not only attach the vesicles to the cytoskeleton but, when intracellular calcium rises, activate the actin polymerisation involved in transport.

Annexin II is phosphorylated in chromaffin cells within a few minutes of stimulation of secretion (Creutz *et al.*, 1987). The *in vitro* consequence of a reduction in membrane aggregating ability suggests that phosphorylation is a switch off mechanism rather than an enabling step. However, it may be that only monomeric annexin II is phosphorylated *in vivo* and the tetramer, which is partially protected by the proximity

of bound p11 to the phosphorylation sites, is not affected. Alternatively, since a dephosphorylation event has been implicated in exocytosis by some experimental observations (Botana and Macglashan Jr., 1993; Jena *et al.*, 1991), the increase in annexin II phosphorylation could be part of a phosphorylation/dephosphorylation cycle. However, this is unlikely given the observation by Sarafian *et al.* (1991) that thiophosphorylated annexin II (using ATP-γ-S), is more active in reconstituting secretion from permeabilised chromaffin cells than annexin II phosphorylated by ATP.

Recently other proteins have been implicated in vesicle targeting and fusion. An N-ethyl maleidimide-sensitive factor (NSF) is necessary for vesicle fusion with target membranes in many systems, along with soluble NSF-attachment proteins (SNAPs), required to attach the protein to the membrane. The SNAPs bind to specific SNAP receptors (SNAREs) which are either integral membrane proteins or attached by post-translationally added fatty acyl chains; recognition of a specific SNARE on the vesicle membrane by a complimentary SNARE on the target membrane is postulated to provide the specificity (Rothman and Warren, 1994 for a review). Various SNAP receptors (SNAREs) have been isolated and identified specifically associated with either vesicle or plasma membrane. These include: synaptobrevin from synaptic vesicles; the pre-synaptic plasma membrane-specific SNAREs, syntaxin and SNAP-25 (confusingly, this acronym stands for an unrelated synaptosome-associated-protein of 25 kD); and a widely expressed synaptobrevin homologue called cellubrevin. These have all been identified as targets of Clostridial neurotoxins. An NSF-SNAP complex can bind synaptobrevin, and syntaxin and SNAP-25, and it is predicted that interaction between synaptobrevin and the latter two, facilitated by NSF-SNAP, allows docking or fusion of synaptic vesicles. It is difficult to envisage how annexin II might interact with this system which seems to be sufficient for fusion of vesicles. Perhaps this indicates that the role of annexin II lies not at the exocytotic event but upstream.

6.5 Annexin II and endocytosis

While the work in this thesis was in progress, several papers emerged implicating annexin II in endocytosis (Emans *et al.*, 1993; Harder and Gerke, 1993).

Annexin II has been localised to early endosomal vesicles in BHK cells suggesting that the punctate staining seen in the cytosol of RBL-2H3 cells could be endosomal vesicles. However the central location of the putative vesicles seen in RBL-2H3 cells is more consistent with them being late endosomal vesicles. Further studies involving annexin II depression in RBL-2H3 cells should also examine the effects of this on endocytosis. A role for annexin II in endocytosis is discussed further in section 6.7.

6.6 Annexin II and growth

Annexin II is a primary response gene induced in quiescent fibroblasts in response to stimulation by several growth factors (Keutzer and Hirschhorn, 1990); its expression has been shown to increase during the G1 to S phase transition in CHO cells (Chiang *et al.*, 1993). Immunofluorescent localisation of annexin II (figure 3.6) clearly demonstrated that very different levels of annexin II can exist in different RBL-2H3 cells within a population. These differences were often observed in adjacent cells which probably descended from the same parent cell. In addition to this no evidence of such marked clonal variation in annexin II levels was found (figure 3.5) making it unlikely that such large differences exist permanently. This indicates that annexin II levels probably alter over time in these cells and this is likely to be related to the stage of the cell cycle. This was to some extent confirmed by the analysis of annexin II levels in the transfected clones in relation to their degree of confluence, i.e. annexin II levels in the population as a whole do change and are lowest when the cells are confluent and not growing (figure 5.14). There is another possible explanation that depends on the ability of the antibody used for immunolocalisation (HH7) to detect tetrameric annexin II, remembering that the N-terminus against which it was raised is partially covered by p11 in the tetramer. It has not been demonstrated that HH7 does recognise tetrameric annexin II, so if this antibody only binds to monomeric annexin II then the differences in annexin II detected may instead reflect differences in p11 expression and thus alterations in the proportion of monomeric to tetrameric annexin II.

The enlarged cells labelled by immunofluorescence in figure 3.6 D show strongly increased annexin II expression. The subcellular distribution is altered in these large

cells: the extra annexin II is largely cytosolic, which indicates that p11 expression has not been coordinately increased since this should recruit annexin II to the cytoskeleton. Could the increase in annexin II expression have a causal role in their increased size?

Roles in growth and exocytosis could be reconciled if secretion of an autocrine growth hormone is involved in RBL-2H3 cell growth and division. Thus perhaps synthesis of all components of the constitutive secretory pathway are upregulated during cell growth and division. Although the constitutive secretion pathway is not governed by calcium and therefore annexin II is an unlikely participant, the calcium-independent forms could function in this pathway.

If annexin II plays an essential part in cell growth or division there will be very strong selective pressure on cells expressing antisense annexin II to lose the antisense insert, or upregulate transcription from their endogenous gene. Reversion of clones carrying the constitutively expressed annexin II cDNA (in both orientations) indicates that there is selective pressure on RBL-2H3 cells to express "normal" levels of annexin II. This does not confirm that annexin II is involved in the cell cycle, only that cells appear to have a growth advantage if they have normal annexin II levels.

Cells have many different responses to calcium, these vary between cell types and not all are invoked at any one time. Therefore a cell's responses must be pitched to trigger at certain calcium levels and these probably vary between cell types. It is possible that different cell types could have recruited different members of the annexin family to perform the same function but in response to different calcium levels (different annexins have evolved with different calcium sensitivities), or that are regulated by different factors (for example phosphorylation and p11 or calcyclin binding).

On the other hand most cells express more than one annexin and, just like EF-hand-containing proteins, each may have unique or multiple functions of its own. In particular, those annexins with very long N-termini may have adopted the core region simply as a calcium-sensitive domain responsible only for attachment to membranes.

6.7 RBL-2H3 cells as a model system for studying exocytosis

RBL-2H3 cells and mast cells show some important differences in their secretory characteristics. Permeabilised RBL-2H3 cells stimulated to secrete by calcium and non-hydrolysable guanine-nucleotide analogues require ATP in the permeabilising medium. The reasons for this are not known but it suggests that in RBL-2H3 cells some component of the secretory apparatus needs to be phosphorylated after stimulation. This difference between mast and RBL-2H3 cells could be the result of any one of a number of possible differences: there may be extra steps involved between stimulation and the exocytotic event in RBL-2H3 cells which require ATP; or a more fundamental difference in the pathway leading to exocytosis. Permeabilised mast cells do not require ATP if stimulated immediately after permeabilisation, however if more than 30 seconds elapses between permeabilisation and stimulation, mast cells become dependent upon mM ATP (Howell *et al.*, 1989). This suggests perhaps that a basal phosphorylation state is maintained in unstimulated cells which is lost within 30 seconds. ATP enhances the affinity for calcium in mast cells under these circumstances; whether ATP is necessary for the same function in RBL-2H3 cells is a matter for speculation.

The time course of secretion is different for permeabilised mast and RBL-2H3 cells. Mast cells complete secretion in under 2 minutes at pCa5-6 (under 6 minutes at pCa7 and below) (Lillie and Gomperts, 1992), while in RBL-2H3 cells, release occurs over 20 minutes. RBL-2H3 cell secretory vesicles are located in the perinuclear region (De Matteis *et al.*, 1991), so transport of vesicles to the plasma membrane and docking there could contribute to their longer release time and, perhaps, their ATP requirement.

6.8 Annexin II homology with proteins of the rab family

Rab proteins are monomeric GTP-binding proteins of the *ras* superfamily, with M_r of approximately 21kD. The rab family includes the YPT1 and SEC4 gene products necessary for exocytosis in yeast. Rabs become membrane-attached via geranyl-geranyl units attached during post-translational modification. Most vesicles and organelles of both the secretory and the endocytotic pathways carry at least one member of the rab family of proteins on their surface, where they are believed to be involved in trafficking

and targeting of intracellular vesicles (Balch, 1990 for a review). Individual members have been implicated in both exocytosis (for example inhibition of rab3b expression inhibits calcium-dependent exocytosis in rat anterior pituitary cells (Lledo *et al.*, 1993)) and endocytosis (rab9 functions in late endosome to trans-Golgi network transport (Lombardi *et al.*, 1993)). This is an interesting parallel with annexin II.

When the N-terminal sequences of several members of the rab family are aligned, a previously unreported homology is observed with the N-terminus of annexin II. In figure 6.4 the amino acids encoded by the N-terminal exon of annexin II are aligned with those of a number of rab family members.

```
rab1                                M S S M N P E Y D Y L F K L L L I G D
rab2                                      M A Y A Y L F K Y I I I G D
rab3a       M A S A T D S R Y G Q K E S S D Q N F D Y M F K I L I I G N
rab3b       M A S V T D G K H G V K D A S D Q N F D Y M F K L L I I G N
rab4                                  M S E T Y D F L F K F L V I G N
rab4b                                 M A E T Y D F L F K F L V I G S
rab5            M A N R G A T R P N G P N T G N K I C Q R K L V L L G E
rab6                      M S T G G D F G N P L R K F K L V F L G E
rab7                                  M T S R K K V L L K V I I L G D
rab8                                  M A K T Y D Y L F K L L L I G D
rab9                                    M A G K S S L F K V I L L G N
rab10                               M A K K T Y D L L F K L L L I G D
rab11                           M G T R D D E Y D Y L F K V V L I G D
YPT1                                  M N S E Y D Y L F K L L L I G N
YPT3                                M C Q E D D Y D Y L F K T V L I G D
SEC4            M S G L R T V S A S S G N G K S Y D S I M K I L L I G D
H-ras                                             M T E Y K L V V V G A

AnxII                                 M S T V H E I L C K L S L E G D
```

Figure 6.4 *Alignment of the first 15 amino acids of the N-terminus of annexin II with the N-termini of members of the rab protein family*
The rab protein sequences apart from rab9 are from Steele-Mortimer *et al.* (1994), rab9 is from Lombardi *et al.* (1993). Invariant residues are in bold type.

The invariably conserved lysine and glycine residues in the rab proteins, shown in bold in figure 6.4, are present in the N-terminus of annexin II from all species (refer to figure 6.3). Rab 8 and rab 10 are 40% identical to annexin II over the 15 residues in this region (ignoring the start methionine), and in a further 20% of the region, substitutions between rab 10 and annexin II are conservative. This is as high as the sequence identity between some other members of the rab family, for example rab 3a and rab 10. However, there are some notable differences: the presence of a glutamic acid

on the N-terminal side of the conserved glycine in annexin II is in a position that is without exception uncharged and non-polar in the rab family.

The rab family N-termini, as aligned here, vary in length from 7 (rab 2) to 22 (rab 3a and 3b) residues 5' of the invariant lysine, however 5 of the 17 listed are of identical length to annexin II (rabs 4, 4b, 7 and 8 and YPT1). The first 15 amino acids of annexin II are encoded by a single exon. However this region of variable length in the rab family is only part of the first coding exon. This weakens the case for a common ancestor and suggests that the similarity is a result of convergent evolution. The invariant lysine has no obvious structural role and the reason for its conservation is unknown, but the invariant glycine is part of the GTP-binding domain (Valencia *et al.*, 1991). Annexin II does not share any other of the conserved GTP-binding residues.

The presence of this rab homology region (rhr) motif in a variety of protein sequence contexts is consistent with the insertion of two amino acids adjacent to this region in rat annexin II not being detrimental to its function.

6.9 Possible functions of the region of rab homology in annexin II

Rab proteins interact with GTPase activating factors (GAPs) through a well-defined "effector" domain (Sigal *et al.*, 1986). The guanine nucleotide- and Mg^{2+}-binding residues (Wittinghofer *et al.*, 1991) and the region responsible for membrane-attachment are known (Kinsella and Maltese, 1991). Studies with chimeric proteins demonstrated that fewer than the last 34 residues of the highly variable C-terminus are sufficient for targeting to the correct membrane (Chavrier *et al.*, 1991). The presence of a region of homology between proteins implicated in the same cellular processes is likely to be more than a coincidence: could annexin II and the rab proteins interact with the same or related cellular component(s) through their similar N-terminal region, possibly a component involved in vesicle trafficking? The inclusion of the conserved glycine of the GTP-binding domain, which normally interacts with the γ-phosphate group, does of course make this region less likely to be involved in interaction with other factors as presumably this would be incompatible with binding GTP. However,

interaction of this glycine residue with another factor could discriminate between GTP and GDP binding, providing a novel control mechanism.

Although rabs have been implicated in targeting endocytic and secretory vesicles, they appear to lack the required specificity (i.e. there can be more than one present on a vesicle and a single rab can be located on more than one type of vesicle) and no protein receptor for rabs, specific for particular membranes, has been identified. Other proteins implicated in vesicle targeting, the NSF-SNAP-SNARE complexes, are postulated to provide the specificity (Rothman and Warren, 1994). Within this hypothesis, rabs are suggested to determine the direction of traffic, i.e. to ensure that a vesicle fuses with its target membrane rather than with the membrane from which it was derived. Whether, or how, rab proteins interact with SNAPs and SNAREs is unknown, but if the N-terminus is involved then it may share this ability with the related sequence in annexin II. Interaction of the rab homology region (rhr) of annexin II could target annexin II to the correct membrane, and in particular, localise the protein to the region of membrane contact dictated by the SNARE complex.

In the annexin II_2p11_2 tetramer the rhr will be at least partially occluded by bound p11, which would probably render it inaccessible for interaction with other proteins. Therefore, only monomeric annexin II is likely to be able to interact with other proteins via this domain. This raises the possibility of different roles for monomeric and tetrameric annexin II: the annexin II_2p11_2 tetramer, with its much longer half-life and characteristic attachment to the sub-membraneous cytoskeleton may have an entirely different role from cytoplasmic, monomeric annexin II, possibly involving the rhr. Monomeric annexin II requires mM Ca^{2+} to aggregate membranes *in vitro* and so probably cannot perform this in the cell. However if annexin II is attached calcium-independently to early endosomal membranes with the calcium-dependent membrane-binding surface still available, then the monomer could bind a second membrane (at sub-μM Ca^{2+}) and thus may have a role in fusing endosomal membranes back with the plasma membrane (recycling receptors back to the cell surface) or to other organelles of the endocytic pathway, much as in exocytotic vesicle fusion. Alternatively if calcium-independent annexin II is bound via its normal membrane binding surface,

the N-terminus, the rhr, will be exposed and could interact with other cellular components. Competition between annexin II (present on early endosomes) and a rab protein for a target that interacts with the rhr could determine whether endosomes are sent back to the cell surface or on through the endocytic pathway to the lysosomes.

Transient expression of a chimeric protein containing residues 1-18 of annexin II attached to a complete p11 molecule, in MDCK cells, caused early endosomes to detach from the sub-membraneous area and move further into the cytoplasm of the cell (Harder and Gerke, 1993). The authors propose that the trivalent chimera (it has two binding sites for p11 and one for annexin II) forms large aggregates with the endogenous annexin II and p11. These aggregates, which are localised to the same region of the cells as the detached endosomes, consist of both endogenous annexin II and transfected chimera. Although this strongly implicates annexin II in early endosomal localisation, it does not distinguish between an effect caused by disruption of the tetramer and that of loss of monomeric annexin II, or another alternative, that the chimera competes for some component(s) that binds to the rhr region of annexin II (although if the chimera forms complexes as stable as wild type annexin II and p11 do, there will be few unoccupied rhr domains). Given that the tetramer is extremely stable and has a half-life of 40-50 hours compared to 15 hours for the monomer (Zokas and Glenney, 1987) (implying that it does not exchange with the monomeric pool), in these transient transfection studies the chimera will not be able to disrupt existing tetramer. The authors do not indicate how long after transfection their observations are recorded but it seems likely that they are observing the effects of loss of monomeric annexin II rather than interruption of the tetramer (although it appears to have the effect of causing the tetramer to relocate). Thus it is the monomer that is the more likely participant in early endosome localisation.

Annexin II appears to be made up of a patchwork of domains that are shared by other proteins. Attention has previously focused on the homology between the C-terminus of annexin II and a region of 14-3-3 proteins (Roth *et al.*, 1993). This region extends over 16 amino acid residues and contains homology of similar magnitude to that shown with rabs (see below).

Annexin II N-terminus	M S T V H E I L C K L S L E G D . . .
Rab 10 N-terminus	M A K K T Y D L L F K L L I G D . . .

Annexin II C-terminus	. . . K G D Y Q K A L L Y L D G G D D
β 14-3-3 (internal region)	. . . K G D Y F R Y L S E V A S G D N . . .

A peptide based on the C-terminus of annexin II inhibits chromaffin cell secretion (Roth *et al.*, 1993), but an N-terminal peptide has no effect on exocytosis (Ali and Burgoyne, 1990). These results suggest that the C-terminus interacts with other proteins during exocytosis, but the N-terminus does not. A possible explanation of this is that the tetramer (in which the N-terminus is covered by bound p11), and not the monomer, functions in exocytosis. If this is the case then none of the components of the secretory machinery would recognise the N-terminus of annexin II and peptides corresponding to this region would therefore have no effect on the process. The effect of N-terminal peptides in an endocytosis assay would be interesting and could indicate whether the monomer has a role of its own in this process.

6.10 Conclusions

This thesis contains some interesting results. Firstly, the possible alternative splicing of the rat annexin II N-terminus shown here may prove to be a novel regulatory mechanism for this protein. Secondly, the localisation of annexin II in RBL-2H3 cells is similar to that observed in other cell types and is consistent with a role in exocytosis (or endocytosis). Thirdly, the reduction in secretory ability shown by an RBL-2H3 clone with reduced levels of annexin II expression supports the hypothesis that annexin II functions in secretion. Finally, the observation that the return of annexin II expression to wild type levels was concurrent with the return of the secretory response to normal also implicates annexin II in exocytosis. Continuation of this work using an expression vector with an inducible promoter to control transcription of antisense annexin II cDNA could prove to be a valuable method for investigating the function of this protein. It is hoped that this approach will enable definitive secretion experiments to be

performed on intact cells with diminished annexin II expression, and provide the first unequivocal assignment of a function to a member of the annexin family.

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(That's enough thankyous. Ed..)

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