SIGNAL TRANSDUCTION BY THE HIGH AFFINITY RECEPTOR FOR IMMUNOGLOBULIN E, FcεR1, IN THE MAST CELL LINE RBL2H3.

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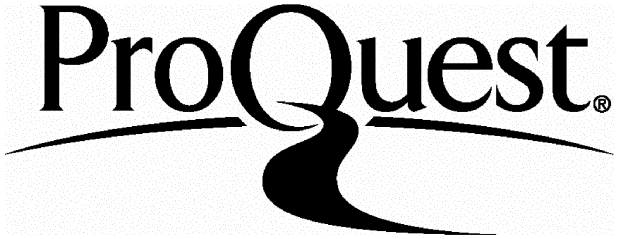

ABSTRACT

Antigenic crosslinking of the FcεR1 on mast cells results in the expression of inflammatory function. A complex mixture of cytokines, chemokines and allergic mediators is released into the context of an inflammatory site. The interplay between these effector molecules is poorly understood, as are the signalling pathways which control their generation. The experiments described in this thesis derived from two proposed ideas. First, that the FcεR1 may regulate adapter molecule cassettes analogous to those which link other antigen receptors to Ras family GTPases and second, that FcεR1 regulation of Ras family GTPases may control aspects of mast cell function.

The FcεR1 regulates components of protein complexes formed around the adapter molecule Grb2. A 33kDa tyrosine phosphoprotein is postulated to be the major adapter between the Grb2 SH2 domain and the FcεR1 ITAMs. The SH3 domains of Grb2 adapt to multiple candidate effector proteins. These include the SLP-76 protein, a newly recognised adapter molecule and the Sos protein, a guanine nucleotide exchange factor for Ras. Protein complexes nucleated around the Grb2 molecule may therefore link the FcεR1 to the regulation of Ras family GTPases.

The Ras GTPase regulates distinct nuclear targets of FcεR1 signals by different effector signalling pathways. FcεR1 regulation of transcription factors is required for induction of multiple genes observed after antigenic crosslinking of the FcεR1 on mast cells. Ras signals are necessary and sufficient for FcεR1 regulation of Elk-1, a transcription factor important in immediate early gene induction. Ras signals to Elk-1 via the Raf-1/MEK/ERK pathway; dominant inhibition of this effector cascade ablates FcεR1 stimulation of Elk-1 transactivation.

Ras is also involved in FcεR1 regulation of the Nuclear Factor of Activated T cells (NFAT) transcription factor. NFAT regulates the promoters of multiple cytokines, growth factors and other immune response genes in a co-operative transcriptional complex with a dimer of AP-1 proteins. FcεR1 induction of Ras is necessary but not sufficient for activation of an NFAT/AP-1 reporter derived from the murine IL-4 promoter and does not involve the Raf-1/MEK/ERK cascade. Dominant inhibition by Ras of NFAT/AP-1 transcriptional activity can be rescued by constitutive activation of Rac-1. These data suggest that regulation of a Rac-1 signalling pathway may be the effector mechanism through which Ras regulates NFAT/AP-1 transcriptional activity.

Rac-1 activity is absolutely required for FcεR1 regulation of NFAT. Rac-1 regulates the subcellular localisation of NFAT protein in RBL2H3. Dominant inhibition of Rac-1, but not Ras, prevents FcεR1 regulated dephosphorylation and nuclear import of NFAT, providing a mechanism for Rac-1 involvement in regulation of NFAT transcriptional activity. This suggests that both Ras dependent and independent Rac-1 signalling pathways contribute to FcεR1 induction of the NFAT/AP-1 transcriptional complex. These are 1) A Ras/Rac-1 pathway postulated to regulate the AP-1 dimer component of the NFAT/AP-1 transcriptional complex and 2) A Ras independent requirement for Rac-1 in FcεR1 regulation of NFAT phosphorylation status and hence subcellular localisation.

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ABBREVIATIONS

ATP	adenosine 5'-triphosphate
BCR	B cell antigen receptor
CAT	chloramphenicol acetyltransferase
CD	cluster of differentiation
CMV	cytomegalovirus
CN	Calcineurin
CRIB	Cdc42/Rac interactive binding
CsA	Cyclosporin A
CTP	Cytidine triphosphate
Cyto D	Cytochalasin D
DAG	*sn*-1,2-diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGTA	[ethylene-bis(oxyethylenenitrilo)] tetra-acetic acid
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FCS	foetal calf serum
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
Grb2	growth factor receptor-bound protein 2
GST	glutathione-S-transferase
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IFN	interferon
Ig	immunoglobulin
IL	Interleukin
IPTG	isopropyl-2-D-thiogalactopyranoside
ITAM	immuno -receptor tyrosine-based activation motif
JNK	Jun N-terminal kinase
MAP	mitogen-activated protein
MHC	major histocompatibility complex

min	minute
mRNA	messenger RNA
Mr	relative molecular weight
NFAT	nuclear factor of activated T cells
NP-40	Nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocyte
PBS	phosphate-buffered saline
PdBu	phorbol 12,13-dibutyrate
PDGF	platelet-derived growth factor
PH	pleckstrin homology
PI	phosphatidyl inositol / propidium iodide (Chapter 7)
PKC	protein kinase C
PLC	phospholipase C
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
pTyr	phosphotyrosine
PVDF	polyvinylidene difluoride
RBL	rat basophilic leukaemia
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
RSV	Rous sarcoma virus
SDS	sodium dodecyl sulphate
SH	src homology
SLO	Streptolysin O
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa
Sos	son of sevenless
SRE	serum response element
SRF	serum rsponse factor
Tc	cytotoxic T cell
TCF	ternary complex factor
TCR	T cell antigen receptor
Th	helper T cell
TNF	tumour necrosis factor
Tris	tris(hydroxymethyl)aminomethane
WASP	Wiscott-Aldrich syndrome protein
ZAP-70	zeta-associated protein of 70 kDa

TABLE OF CONTENTS

CHAPTER ONE

GENERAL INTRODUCTION

1.1 MAST CELLS: bridging innate and acquired immunity.

Mast cells are an immune effector cell population first described by Paul Ehrlich in 1897 as 'mast' (German; well fed), referring to their granule-rich cytoplasmic appearance. They are found in all connective and mucosal tissues which form the body's primary surfaces of contact with antigenic challenge. As part of the acquired immune response mast cells initiate inflammation in response to antigenic challenge; they have surface receptors for the Cε2:Cε3 junction of the Fc region of Immunoglobulin E (IgE). Aggregation of the FcεR1 receptors by the interaction of multivalent antigen with bound IgE results in the rapid extracellular release of histamine, heparin, proteases and other mediators from cytoplasmic granules and in the synthesis and secretion of leukotrienes and prostaglandins (Jouvin *et al.*, 1995; Metzger, 1992, Daeron, 1997). The actions of these mediators result in pro-inflammatory events including increased vascular permeability, changes in blood vessel tone and limited killing function towards a pathogen. Moreover, antigenic stimulation results in the induction of genes for multiple chemokines and cytokines.

Chemokine release results in the formation of a chemotactic gradient up which infiltrating secondary leukocyte populations (taking advantage of the newly increased permability of the vascular wall) can move. The array of cytokines produced by mast cells can be activatory for these infiltrating effector cell populations and can have more systemic roles in orchestration of a broader immune response. In response to mast cell initiation of an inflammatory response, a number of cell types will move into a site of challenge. These include terminally differentiated effector cells such as neutrophils and eosinophils, which use oxidative bursts to eradicate pathogenic organisms. T-lymphocytes will also infiltrate an inflammatory site. Differentiation of these cells towards Th (or Tc) subsets will be influenced by mast cell derived cytokines. The range of activation dependent mast cell products is listed in Figure 1.1.

Mast cellproducts	
Pro-inflammatory cytokines IL-1 IL-5 TNFα,	**Inflammatory mediators** serotonin leukotrienes prostaglandins histamine serine esterases sugar aminidases
Immunoregulatory cytokines IL-3 IL-8 IL-12 IL-4 IL-10 GM-CSF IL-6, IL-13	**Chemokines** MIP-1α MIP-1β

Figure 1.1. Released products of mast cell activation. IL: interleukin, TNF: Tumour necrosis factor, GM-CSF: granulocyte/macrophage colony stimulating factor, MIP: macrophage inflammatory protein. References: (Bressler *et al.*, 1997; Mecheri and David, 1997; Plaut *et al.*, 1989).

There is a clear role for mast cells in natural or innate immunity. It is well established that mast cell driven inflammation can be initiated by non-immunological bacterial products such as lipopolysaccharide. Moreover, mast cell activation can act to amplify features of acute non-specific immune responses such as the recruitment of neutrophils. Two papers in 1996 reported that mast cells represent a central component of host defence against bacterial infection. These studies used reconstitution of mast cell deficient mice to show that mast cell promote clearance of bacterial infection and that this effect is apparently dependent upon mast cell release of TNFα; administration of anti-TNFα ablates the curative effect of mast cell introduction (Echtenacher *et al.*, 1997; Malaviya *et al.*, 1996).

These data show that mast cells are important components of innate immunity. By virtue of their ability to interact with other immune cell types either directly (via surface receptors) or indirectly (via cytokines/chemokines), mast cells are also important orchestrators of the later acquired immune response to challenge (Mecheri and David, 1997). Mast cells express MHC class II molecules and are capable of processing and presenting antigenic peptide to $CD4^+$ T cells. Moreover, mast cells express surface markers which are co-stimulatory for T Cells (B7-1, B7-2, CD40) and B cells (CD40 ligand). Mast cells subsets which express low levels of MHC class I may be able to influence CD8+ cytotoxic T cell responses to bacterial antigens (Malaviya *et al.*, 1997).

The production of two cytokines by mast cells in response to antigen may be of particular significance in the subsquent polarisation of T cell subsets. The fact that

CD4+ T helper cells (Th) can be functionally distinguished on the basis of the profile of cytokines which they produce is known to be relevant to the type of immune response generated. In certain disease cases polarisation of T cell subsets represents the difference between disease progression and cure. Mast cell production of IL-4 will polarise T cells towards a Th2 phenotype as well as inducing B cell isotype switching to IgE; a major feed-forward mechanism for the protraction of an inflammatory response. IL-12 production by mast cells seems to be dependent on prior exposure to Stem Cell Factor (the c-*kit* ligand) but if produced will drive T cell differentiation to a Th1 phenotype. The postion of mast cells as orchestrators of acquired immunity is shown in Figure 1.2.

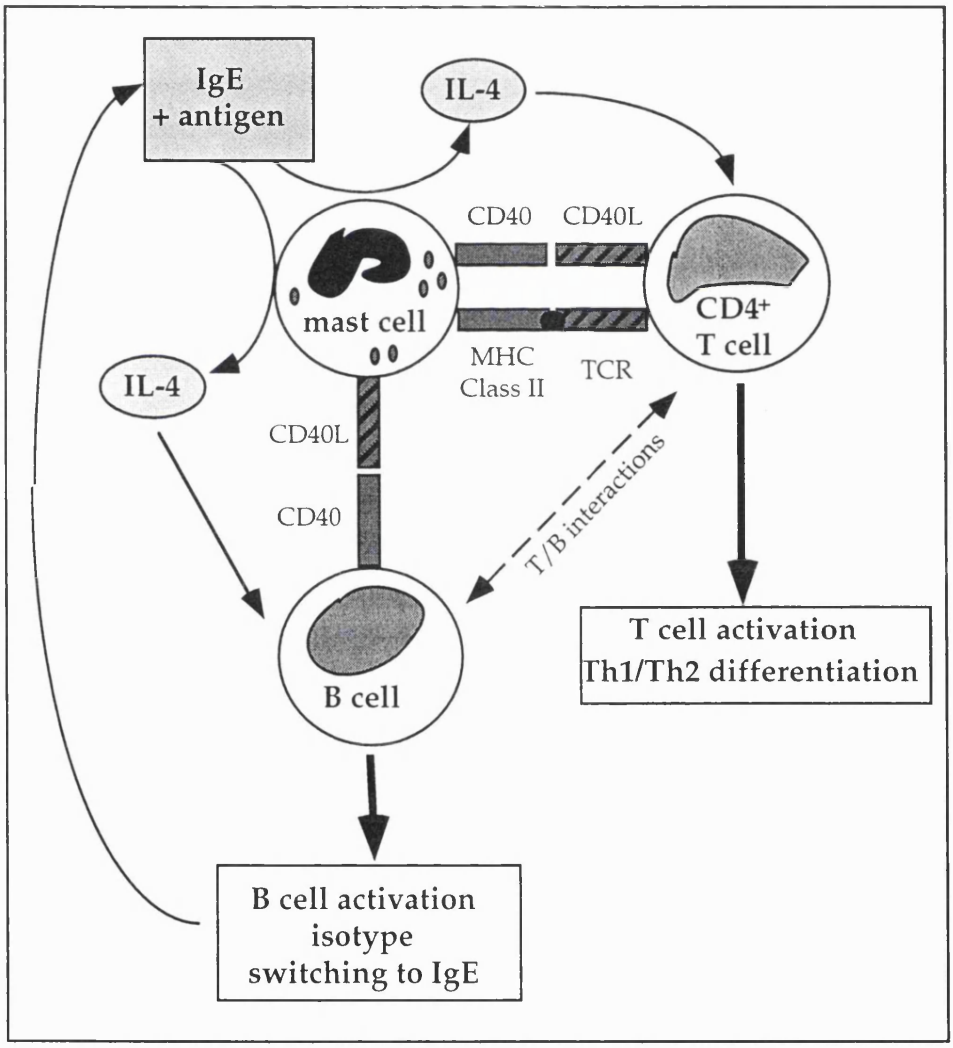


Figure 1.2. Mast cells orchestrate T and B cell commitment decisions in the course on an inflammatory response. Both IgE dependent antigenic exposure and MHC Class II derived signals may activate mast cells to produce IL-4 (Mecheri and David, 1997).

The importance of mast cells as immune effectors is undeniable. The pathology associated with their inappropriate activation is severe. Sudden degranulation of mast cells in response to harmless doses of antigen (eg. bee venom and peanut antigens, penicillin) results in the phenomenon of anaphylactic shock. Anaphylaxis is characterised by intense bronchospasm, vomiting, skin rash/pruritis, oedema of the nose and throat and vascular collapse. Mast cell activation underlies other forms of hyperensitivity. As will be discussed below, the atopic phenotype of recurrent persistent allergic reactions is mast cell derived. Non-anaphylactic chronic allergic reactions such as allergic rhinitis and asthma are caused by contact of allergen (eg. pollen, house dust mite faeces) with mast cell bound IgE in the nasal mucosa, bronchial tree or conjunctiva. These result in the typical symptoms of type I allergic reactions; pruritis, oedema, increased mucus production and bronchoconstriction.

Antigenic crosslinking of the high affinity receptor for IgE, FcεR1, is a major mechanism for mast cell activation. Activation by the FcεR1 is sufficient to induce the full complement of mast cell functional responses (reviewed in (Daeron 1997; Jouvin *et al.*, 1995).). The FcεR1 is one of a vast number of transmembrane receptors which influence all aspects of cellular behaviour in response to ligation. The field of signal transduction study has arisen because of the need to understand mechanisms for intracellular transmission of information. In view of their roles and pathology, this need is well illustrated in the case of mast cells. The activatory signals delivered by the FcεR1 are poorly understood and their further elucidation and functional consequences represent the major focus of the work presented in this thesis.

1.2 The high affinity receptor for Immunoglobulin E, FcεR1.

The FcεR1 is tetrameric (Daeron, 1997; Metzger, 1992; Ravetch and Kinet, 1991). The 60kDa α chain is ligand binding and contains two extracellular C2-type immunoglobulin domains. The α chain has 6 sites of N-linked glycosylation and a short 17aa C-terminal cytoplasmic domain which contains no recognised signalling motifs. Truncation of the FcεR1α cytoplasmic tail does not affect signalling (Alber *et al.*, 1991). The transmembrane region of FcεR1α is highly conserved. Mutation of a specific basic residue in the transmembrane domain ablates interaction with an Aspartate in the FcεR1γ transmembrane domain and hence the ligand dependent signalling potential of the FcεR1 as a whole (Cosson *et al.*, 1991). The FcεR1β chain is a 30kDa molecule containing four transmembrane regions with both the N- and C-termini in the cytoplasm. FcεR1γ is identical to the γ subunit of CD16 and is a disulfide linked homodimer of 20kDa. FcεR1 binds to monomeric IgE through a 76aa region at the Cε2:Cε3 junction in IgE. The stoichiometry of this interaction is 1:1 and the binding affinity is $1x10^{-10}$M (Metzger, 1992). Whilst αβγ2 is the most common

form of the receptor, αγ2 receptors exist on monocytes and some mast cells and can mediate cellular activation events (Alber *et al.*, 1991).

The C-terminus of FcεR1γ and the cytoplamic domains of FcεR1γ contain sequences designated Immunoreceptor Tyrosine-based Activation Motifs (ITAM) (Reth, 1989; Weiss, 1993; Weiss and Littman, 1994, Daeron, 1997). On this basis the FcεR1 is considered part of the leukocyte antigen receptor superfamily along with the T and B-cell antigen receptors and the high affinity IgG receptor, FcγR1. This relationship is illustrated in Figure 1.3.

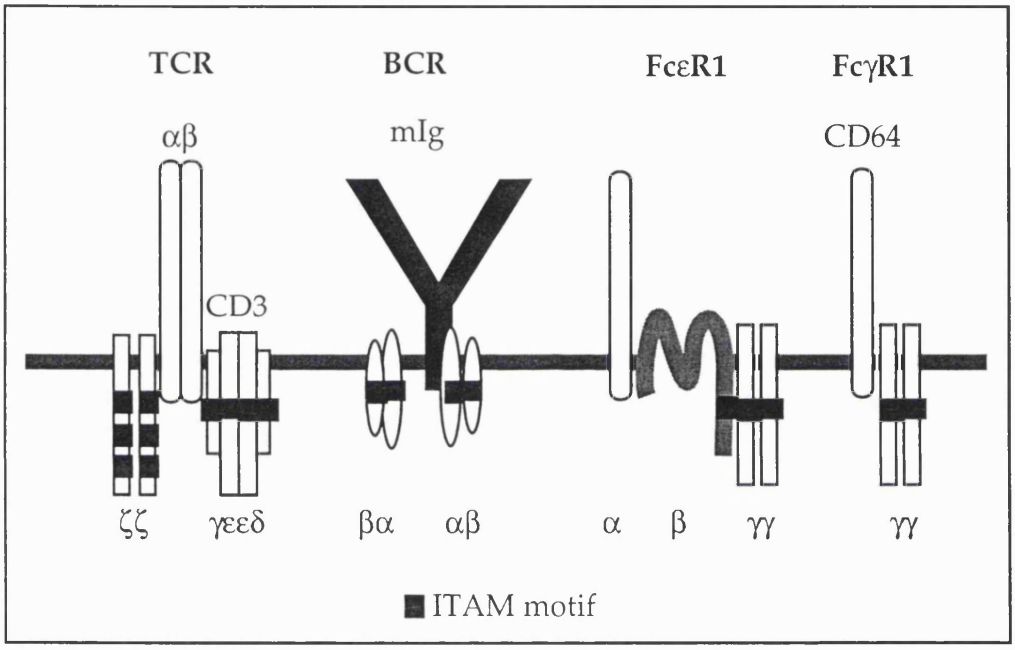


Figure 1.3. The Leukocyte Antigen Receptor Superfamily. Defined by the presence of signalling chains containing the tandem YXXL phosphoacceptor Immunoreceptor Tyrosine based Activation Motif (ITAM).

Mast cell deficient mice cannot raise anaphylactic reactions and are defective in their ability to raise inflammatory responses to allergenic challenge. These studies have been refined by the selective knockout of FcεR1 subunits. There have been two informative targeted gene disruption studies concerning FcεR1 subunits. Mice deficient in FcεR1α were generated by the Kinet laboratory (Dombrowicz *et al.*, 1993). These mice cannot raise an anaphylactic reaction in response to cutaneous or systemic challenge. Moreover they show increased expression of FcγRII and FcγRIII which can function as low affinity receptors for IgE, but no compensatory effect is observed, suggesting that these receptors cannot drive anaphylaxis in isolation. FcR γ chain knockout mice (Takai *et al.*, 1994) showed impaired IgE dependent anaphylaxis and also suggested a central role for the FcR γ chain in the initiation of immune-

complex mediated inflammatory responses. These data reflect the dual role of FcR γ as a component of both IgE and IgG receptors.

Antigen receptors govern cell fate through their control of intracellular biochemical signal transduction pathways. Such signalling pathways link the plasma mambrane to cytoplasmic and nuclear targets, overcoming cellular compartmentalisation to allow the membrane bound receptor to exert a remote influence in response to ligation. Understanding the relevant signalling pathways thus translates to undertanding how the FcεR1 regulates mast cell function. In the following section FcεR1 signalling as it is currently understood will be reviewed.

1.3 Signal transduction by the FcεR1; current knowledge.

1.3.1.The ITAM motif and FcεR1 interactions with cytoplasmic protein tyrosine kinases.

The ITAM is composed of D/E-XX-YXXL-X_{7-11}-YXXL-L/I (Reth, 1989). These Tyrosine residues are phosphoacceptor sites for the action of receptor associated protein tyrosine kinases (PTKs). Mutation of the Tyr residues in the β and γ ITAMs ablates the signalling potential of the FcεR1 and functional responses to antigen such as degranulation and cytokine gene induction. ITAM Tyr residues are phosphorylated within 30 sec of antigenic crosslinking of the FcεR1, this event is reversed by the addition of monovalent hapten (Paolini *et al.*, 1991). Multiple tyrosine phosphorylation events follow FcεR1 ligation (Benhamou *et al.*, 1992). There are two species of FcεR1 associated tyrosine kinases; the *src* family kinase lyn (Eiseman and Bolen, 1992; Jouvin *et al.*, 1994) and the p72Syk kinase (Benhamou *et al.*, 1993; Shiue *et al.*, 1995). The former is found associated with FcεR1β, the latter is able to bind β and γ but has higher affinity for interaction with FcεR1γ. The early aggregation and phosphorylation events following FcεR1 ligation that involve these two kinases are described below.

The *src* family of mammalian PTKs has eight members (Bolen and Brugge, 1997). In addition to a catalytic domain, *src* kinases possess a range of domains involved in protein-protein interactions. The nature of these *src* homology (SH) domains and other structural modules in signalling proteins will be discussed in detail later. In addition, *src* family members possess a C-terminal phosphorylation site which is able to in part regulate their activity. Most *src* kinases exhibit autophosphorylation. An important distinction between *src* kinases and other non-receptor tyrosine kinases is their constitutive membrane association. This is mediated by an N-terminal SH4 domain (Resh, 1994). The relevant *src* kinase in FcεR1 signalling is lyn (Eiseman and Bolen, 1992). This 54kDa PTK is localised to the

plasma membrane by a myristoylated glycine residue in its N-terminus and is associated with FcεR1γ in resting cells.

1.3.2 Cis- and trans-phosphorylation events follow FcεR 1 crosslinking and aggregation by multivalent antigen.

Antigenic crosslinking of the FcεR1 results in receptor aggregation within the plane of the plasma membrane. This aggregation is sufficient to activate lyn which autophosphorylates. Once active, lyn transphosphorylates ITAM motifs within adjacent clustered FcεR1 (Pribluda *et al.*, 1994). It is well established that aggregation of ITAM bearing cytoplasmic domains of signalling proteins is sufficient to induce signalling. In a series of important experiments chimeric receptors were made of CD8 or IL-2Rα extracellular domains and the cytoplasmic tails of FcεR1β (C-terminus, ITAM containing) or FcεR1γ (Letourneur and Klausner, 1991; Eiseman and Bolen, 1992a). On aggregation the FcεR1γ chimerae activated lyn and Syk, induced secretion and caused a modulation in $[Ca^{2+}]_i$, presumably via PLCγ1 activation. Lyn associated with FcεR1β chimerae but not FcεR1γ, the converse being true for Syk. Aggregation of FcεR1β chimerae did not activate lyn or Syk and did not induce any functional response such as secretion (Wilson *et al.*, 1995; Jouvin *et al,* 1994). These data lead to FcεR1β being regarded for a time as a possible intrinsic down-regulator of FcεR1 signalling (Wilson *et al.*, 1995).

Phosphorylated ITAMs act as docking sites for the SH2 domains of the Syk kinase. As will be discussed, SH2 domains have high affinity for phosphorylated tyrosine residues. Accordingly, Syk is recruited to the FcεR1 complex, where it is a substrate for lyn and, once activated in this fashion, autophosphorylates. These data come from mast cell biochemistry and also the following reconstitution experiments in fibroblasts (Scharenberg *et al.*, 1995). NIH-3T3 bearing stable expression of FcεR1α and β and γ were generated. Co-expression of lyn and Syk either singly or in concert was then carried out using recombinant vaccinia viruses. A kinase-deficient Syk mutant was phosphorylated equivalently to wild-type Syk in this system upon FcεR1 aggregation. However, this only occurred in the presence of co-expressed lyn; indicating that lyn is the Syk-kinase.

Syk activation initiates a plethora of signalling events. The importance of Syk in the initiation and control of mast cell functional events is illustrated by data from Syk knockout mice (Costello *et al.*, 1996; Turner *et al.*, 1995b).. Syk-/- mice do not raise passive cutaneous anaphylactic reactions to antigenic challenge. Mast cells isolated from these mice show marked decreases in FcεR1 induced tyrosine kinase activity and do not secrete allergic mediators or modulate $[Ca^{2+}]_i$ in response to FcεR1 crosslinking The importance of syk, the apparent ability of FcεR1α,γ/syk to act as an autonomous signalling unit and the observation that src kinases may not need to be physically

receptor associated raise interesting questions as to the role of the FcεR1β chain. As described above, chimeric receptor experiments lead to the hypothesis that if not actually a negative regulator, FcεR1 β was not a positive contributor to FcεR1 signalling (Wilson *et al,* 1995). On the basis of some limited sequence homology, FcεR1β was even regarded briefly as a putative calcium channel. The reputation of FcεR1β has been rehabilitated recently by the work of the Kinet laboratory.

Lin *et al* (Lin *et al.*, 1996) compared the signalling capabilities of wild-type and mutant αγ2 and αβγ2 FcεR1 complexes in fibroblast and monocyte reconstitution experiments. The data show that in comparison with αγ2, αβγ2 triggered cells have 1) enhanced lyn dependent phosphorylation of γ, 2) enhanced syk kinase activity and tyrosine phosphorylation and 3) enhanced mobilisation of $[Ca^{2+}]_i$. Therefore the β chain of the FcεR1 functions as a signal amplifier. Quantitation of the increased tyrosine phosphorylation and $[Ca^{2+}]_i$ mobilisation in αβγ2 vs αγ2 triggered cells showed that the 'gain' provided by the β amplifier was approximately 5-7 fold. These data are of particular interest given the linkage between atopy and polymorphisms in FcεR1β.

Atopic disease is associated with high circulating levels of IgE and presents multiple concurrent allergic reactions. Severe atopic disease is familial and is associated with polymorphisms in the FcεR1β chain. (Hill *et al.*, 1995b; Shirakawa *et al.*, 1994b) Loci for atopy and FcεR1β co-localise upon chromosome 11q (Shirakawa *et al.*, 1994a). The requirement for analysis of the contribution of β polymorphisms to pathology has driven extensive study of the role of the FcεR1β chain in mast cell activation. A basic hypothesis is that alterations in conformation conferred by the atopic mutation results in an enhanced gain and hyper-responsiveness to IgE/antigen dependent activation of mast cells. In mast cells from atopic individuals the threshold for IgE/antigen dependent activation may be lowered.

This section has examined the sequence of events following FcεR1 crosslinking in the immediate receptor microenvironment. Antigenic crosslinking of the FcεR1 results in activation of a number of intracellular signalling pathways for the transmission of information to effector targets. A review of some of those signals will now follow. Certain signals which are common with other systems will be reviewed briefly, followed by an examination of the signalling cassettes which comprise GTPase/kinase cascades and their relevance to antigen receptor signalling. However, it is important to note that there is an excess of characterised target molecules for FcεR1 regulated kinases over defined signalling cassettes into which they can be placed. For clarity and brevity, some of these PTK targets in the FcεR1 system are described in Figure 1.4, along with comments on their putative functions.

Substrate	Mw (kDa)	Domains	Comments	Ref
Btk	60	PH, TecH SH2, SH1	Mutation at locus onXq22 underlies X-linked agammaglobulinemia. Poss. role in apoptosis, downstream effectors uncharacterised.	a
Itk(Tsk/Emt)	65	PH, TecH SH2, SH1	Itk-/- mice have decreased thymocyte numbers and low mature Tcell proliferative responses. Unknown function in mast cells.	b
p125 FAK	125	Integrin BD, SH1, Paxillin BD	Focal adhesion kinase. Regulation of FA components inc. Paxillin, Talin. Connection to Rho GTPase signaling in fibroblasts. Mast cell FA formation and increasedadherence to substratum follows FcεR1 ligation.	c
FAKAP	77	ND	FAK associated protein, identified in mast cells. Tyrosine phosphorylated after FcεR1 crosslinking.	d
Fer	95	SH2, SH1	Growth factors and FcεR1 induce Fer tyrosine phosphorylation. Associates indirectly with cadherin/catenin, thus involved in cytoskeletal regulation. Also a nuclear pool of this kinase.	e
Cbl	120	RING finger Pro rich Basic PTB	Pluripotent adapter molecule associates with ZAP-70, PI 3-kinase p85 sub-unit and multiple other adapters. May play a role in negative regulation of antigen receptor signaling	f
Vav	95	DH, PH Cys rich SH2, SH3	GEF for Rac-1/CDC42 GTPases. Tyr phosphorylated in response to TCR, BCR, FcεR1 ligation. Vav deficient mice show defective proliferation of T and B cells.	g

Figure 1.4. Substrates for FcεR1 associated PTKs. PH: pleckstrin homology, SH: src homology, TecH: Tec homology, FA: focal adhesion, BD: binding domain, ND: not determined. DH, Dbl homology; RING, Really Interesting New Gene; PTB, Phosphotyrosine binding. References refer to mast cell observations: a) (Kawakami *et al.*, 1994) b) (Kawakami *et al.*, 1995), c) (Hamawy *et*

al., 1993) d) (Hamawy *et al.*, 1995) e) (Penhallow *et al.*, 1995) f) (Ota *et al.*, 1996) (g) (Margolis *et al.*, 1992).

1.3.3 FcεR1 regulation of intracellular free calcium levels.

A major consequence of the antigenic stimulation of all members of the Leukocyte Antigen Receptor Superfamily is the elevation of intracellular free calcium $[Ca^{2+}]i$. FcεR1 ligation induces a biphasic calcium response (Lee and Oliver, 1995; Millard *et al.*, 1988) Firstly, there is a rapid, transient, increase in levels of $[Ca^{2+}]i$. This transient is due to influx of calcium from the environment. This process is mimicked by pharmacological ionophores such as Ionomycin, which effectively open cells to environmental levels of calcium. The second phase of the calcium flux induced by the FcεR1 is a sustained elevation in $[Ca^{2+}]i$ due to the mobilisation of intracellular calcium stores from the endoplasmic reticulum. This latter event is mimicked by agents such as Thapsigargin which inhibit the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) pump normally responsible for filling intracellular stores. Examination of this phase at the single cell level suggests that it may comprise a series of regular oscillations, where there is periodic release of calcium from intracellular stores (Millard *et al.*, 1988).

On FcεR1 aggregation, Phospholipase Cγ1 (PLCγ1) is recruited to the plasma membrane and tyrosine phosphorylated (Park *et al.*, 1991b), possibly by the Syk kinase. Activated PLCγ1 catalyses the hydrolysis of the membrane inositol phospholipid PI 4,5-P_2 to generate the second messengers Inositol 1,4,5 tri-phosphate (IP3) and diacylglycerol (DAG). IP3 stimulates the mobilisation of calcium from intracellular stores by binding to IP3 receptors on the surface of the endoplasmic reticulum. A third, related pathway has also been described as part of the FcεR1 induced calcium response. Capacitative calcium entry is a late phase of influx from environment which occurs in response to depletion of the endoplasmic reticulum stores. This has been described in RBL2H3 (Lee and Oliver, 1995).

In mast cells, there is apparently also a PLCγ1-independent mechanism for the mobilisation of intracellular calcium stores. A recent paper (Choi *et al.*, 1996) showed that FcεR1 aggregation activates the sphingosine kinase (SK) which singly phosphorylates sphingosine. Free Sphingosine 1-phosphate was able to induce antigen independent calcium flux. Moreover, a sphingosine analogue which acts as a non-productive SK substrate was able to abolish FcεR1 induced calcium flux without affecting syk kinase activity or IP3 levels. Hence in the FcεR1 case, an SK pathway for calcium mobilisation may be of greater importance than the conventional IP3 mediated pathway.

IP3 is not the only second messenger to be produced as a result of PLCγ1 activation. The production of DAG results in the activation of Protein kinase C (PKC)

isoforms. PKCs are a large family of related serine/threonine kinases which may be sub-divided on the basis of their regulation by DAG, phorbol esters and other signalling proteins. The particular PKCs involved in FcεR1 signalling have not been mapped. A number of reconstitution studies were performed in the late 1980s-early 1990s which implicated PKCs in the control of secretion in FcεR1 stimulated mast cells (Buccione *et al.*, 1994; Ozawa *et al.*, 1993). It is clear that 1) a combination of PKC activating phorbol esters and ionomycin are sufficient to pharmacologically induce secretion. 2) PKC inhibitors of varying degrees of selectivity do inhibit FcεR1 induced secretion. However, since these data were generated the number of known PKC isoforms has doubled and the work has not been updated. Therefore no precise assignment of roles in FcεR1 signalling for individual PKC isoforms can be made.

1.3.4 FcεR1 activation of lipid kinases/phosphatases.

Hydrolysis of phosphatidylinositol 4,5 bisphosphate by PLCγ1 is not the only FcεR1 regulation of phospholipid metabolism. The FcεR1 is thought to regulate the phosphatidylinositol 3'-hydroxyl kinase (PI 3-kinase) responsible for phosphorylation of inositol lipids on the D3 position (Barker *et al.*, 1995; Yano *et al.*, 1993). Downstream targets for D3 phosphoinositides in mast cells are not known, although it is clear that use of Wortmannin (WMN) ablates the secretory response (Marquardt *et al.*, 1996; Yano *et al.*, 1993). WMN is an inhibitor which targets PI 3-kinase (Arcaro and Wymann, 1993). It is reasonable therefore to expect that some effector mechanism for PI 3-kinase directly impinges upon the control of secretion. There are many published roles for PI 3-kinase in various cell systems which are beyond the scope of this review. The roles of D-3 phosphorylated lipids in signal transduction are begnning to be explored. These lipid species have been shown to bind the Pleckstrin Homology (PH) domain in cytoplasmic signalling proteins and thus regulate their subcellular localisation (Harlan *et al.*, 1994). Prototypical examples of these interactions are in the binding of D-3 phosphorylated lipids to the PH domains of Protein kinase B (Frech *et al.*, 1997) and Rac/Rho GEF (Zheng *et al.*, 1996) molecules. Effector pathways influenced by these recruitment/activation events include the activation of the ribosomal p70S6 kinase and the effects of Rac/Rho GTPases on organisation of the actin cytoskeleton. Clearly FcεR1 activation of PI 3-kinase may have numerous downstream roles in the complement of responses which together comprise mast cell activation.

Cellular levels of phosphorylated lipids are tightly regulated. In response to receptor signals, pools of non-second messenger lipids are rapidly phosphorylated (or de-phosphorylated) to convert them to more significant forms. Conversely, their de- or re-phosphorylation is also a regulatory mechanism. An IgG receptor in mast cells (FcγRIIB) has been shown to regulate activity of an inositol phosphate phosphatase,

SHIP (SH2 containing Inositol Phosphatase). SHIP hydrolyses PI 3,4,5-P_3 to PI4,5-P_2.SHIP co-precipitates with phosphorylated FcγRIIB in mast cells (Ono *et al.*, 1996) and negatively regulates IgG/immune complex dependent inflammatory responses. Another recent paper has placed FcγRIIB/SHIP in a complex with the FcεR1 and suggested a negative regulatory role for SHIP in FcεR1 signalling (Fong *et al.*, 1996).

1.4 GTPases and kinase cascades.

Transmembrane receptors regulate functional events which are spatially and temporally distinct from their immediate microenvironment. For example, any signal which causes a long term change in cell fate must inevitably affect gene transcription. For receptors to exert remote influence, the signalling cascades they employ must have certain characteristics; precision, sensitivity, an ability to overcome cellular compartmentalisation and a capacity to be efficiently downregulated. Across many receptor/response systems it is clear that a number of common patterns emerge in the signalling cassettes which have evolved to fulfil these requirements. A prototypical case (and a major focus of this thesis) is the use of GTPase/kinase cascades to regulate transcription factor targets. In addition to the calcium/PKC pathways described above, such cascades are a major feature of antigen receptor signalling.

1.4.1 Ras family GTPases regulate conserved signalling modules; the Mitogen Activated Protein (MAP) kinase cascades.

The Ras family of GTPases is extensive, comprising over 50 proteins. This superfamily is divided into a number of sub-families including the Ras and Rho -type proteins Multiple aspects of Ras signaling are reviewed ((Boguski and McCormick, 1993; Downward, 1990; Downward, 1996). The former grouping is made up of the four Ras proteins Harvey-Ras, Kirsten-Ras, Neuroblastoma-Ras and R-Ras) and members of the Rap subfamily. The Rho subfamily of GTPases comprises Rho A, Rho B, Rac-1, Rac-2 and CDC42. GTPases of the Ras superfamily receive and react to information on a vast number of eukaryotic cell processes (Chant and Stowers, 1995). They function as binary switches, since they cycle between GTP- and GDP-bound states. GTP bound GTPases are regarded as active, in that they act as transducers of information to effector molecules. The guanine nucleotide binding status of GTPases is tightly regulated. GTP-bound, active, membrane localised GTPases are in a position to modulate diverse cellular processes. The signalling mechanism employed at this point when the eventual target of the GTPase signal is a transcription factor seems remarkably conserved between systems as asignalling pathways based around a MAP kinase cascade. These signalling modules are found in yeast (to date six such modules are identified, including the signalling pathways used to control mating), *Drosophila*, invertebrates (*C. elegans*) and mammalian cells.

MAPK (Mitogen Activated Protein Kinase) cascades are defined as signalling pathways which involve the sequential phosphorylation of serine/threonine or dual-specificity kinases and have been extensively reviewed (Cahill *et al.*, 1996; Cano and Mahadevan, 1995; Davis, 1993). The basic components of this system are 1) a MAP kinase (MAPK), the effector kinase described in Figure 1.5, which directly phosphorylates a transcription factor, 2) a MAPK kinase (MAPKK), which threonine/tyrosine phosphorylates MAPK and 3) a MAPKK kinase (MAPKKK), which serine phosphorylates and hence activates the MAPKK.

In the yeast system, there is an added component of the GTPase/kinase cascade for which no mammalian equivalent has yet been found. The STE5 protein is a scaffold protein in yeast, apparently responsible for stabilising multi-protein complexes (Choi *et al.*, 1994). These molecules are likely to be part of the 'hard-wiring' of signalling pathways, offering increased efficiency and specificity. Mammalian equivalents have not been identified, possibly because of the lack of detectable activation dependent modifications of scaffold molecules. Alternatively, mammalian systems may have evolutionarily overcome the need for scaffolding of signalling proteins through increased efficiency of short term, highly specifc protein-protein interactions.

1.4.2 The 'Classical' Ras/Raf-1/MEK GTPase/kinase cascade.

GTPase/kinase cascade components are described above in general terms. The prototypical example of this type of signalling cassette (it is also the best understood example) is the Ras/Raf-1/MEK cascade (Marais and Marshall, 1996; Marshall, 1994b). Ras is membrane targetted by prenylation (Hancock *et al.*, 1989) and is inactive (GDP loaded) in unstimulated cells. It is activated by the membrane recruitment of the Sos guanine nucleotide exchange factor, which promotes GTP loading of Ras. Ras.GTP is active and promotes activation of the Raf-1 serine/threonine kinase (Marais *et al.*, 1995).

Raf-1 is a dual specificity serine/threonine kinase which connects Ras to the MEK kinase cascade. Ras interaction is mediated by the Raf-1 N-terminus (Warne *et al.*, 1993). Membrane localisation of Raf-1, in the absence of other stimulation, is sufficient to mediate activation of the MEK/ERK pathway (Leevers *et al.*, 1994; Marshall, 1994a). Moreover, recent experiments from the Perlmutter laboratory suggest that dimerisation of Raf-1 independently of membrane localisation is sufficient for activation of the MEK/ERK cascade (Farrar *et al.*, 1996). Raf-1 phosphorylates the Mitogen-activated ERK kinase (MEK) on four serine residues. MEK is activated in this fashion and phosphorylates its substrate, the Extracellular Signal Regulated Kinase (ERK) upon Tyrosine and Threonine.

ERK kinase translocates to nucleus when it is activated. ERK kinases have multiple transcription factor targets which therefore link Ras to the transcriptional machinery. All members of the Leukocyte Antigen Receptor superfamily have been shown to activate the Ras/Raf-1/MEK/ERK cascade (reviewed in (Alberola-Ila *et al.*, 1997; Daeron, 1997; Gold *et al.*, 1993)). Therefore GTPase/kinase cascades should be considered as major links between antigen receptors and gene induction. The Ras/Raf-1/MEK/ERK cascade is prototypical. It is clear however that GTPases other than Ras have their own accompanying kinase cascades. Activity of components of any one these cascades may impinge upon the status of molecules within another cascade. In truth, these overlapping signalling pathways form networks within which diverse outcomes of a single input may be contrived.

1.4.3 GTPase/kinase cascades are interconnected networks with transcription factor targets.

The Mitogen Activated Protein kinases (MAP kinases) are effector kinases in GTPase/kinase cascades. Their consensus substrate motif, Ser/Thr-Pro, is found in a wide range of transcription factor targets. A number of non-transcriptional targets for MAP kinases have now been reported, for example the MAPKAP2 kinase which is involved in insulin signalling and mitogenic responses (Cuenda *et al.*, 1995) Several distinct vertebrate MAPKs have been identified, the best characterised being three species; 1) The Extracellular signal Regulated Kinases (ERKs), 2) the Jun N-terminal kinases (JNKs) and 3) the p38/RK (reactivating) kinase. A number of other MAPK are known but will not be discussed here, for review see (Su and Karin, 1996) and references therein.

Distinct MAPKs are targets for kinase cascades initiated by distinct Ras family members, in response to distinct extracellular stimuli (Cano and Mahadevan, 1995; Marshall, 1994b). A simplified scheme is shown in Figure 1.5. Extracellular stimuli which initate ERK cascades include mitogens (serum, growth factors), TNFα and IL-1. The JNK signalling module was originally characterised as a Stress activated pathway and as such is responsive to heat or osmotic shock, UV irradiation and treatment with protein synthesis inhibitors. These distinctions are not absolute, it is clear that certain stimuli simultaneously act upon multiple MAPK cascades.

It is also clear that certain transcription factor targets of MAPK cascades can integrate multiple MAPK signals. The Ternary Complex Factor (TCF) Elk-1 (see introduction to Chapter 5), is a target for ERK and JNK MAP kinases (Cavigelli *et al.*, 1995; Gille *et al.*, 1995a; Gille *et al.*, 1995b; Whitmarsh *et al.*, 1995)). ATF-2 is a c-*fos* and TNFα gene regulating transcription factor which is a substrate for both JNKs and the p38/RK MAP kinase (Gupta *et al.*, 1995; Raingeaud *et al.*, 1995). As indicated in Figure 1.5, components of the individual cascades cross-talk. For

example, MEKK1 can phosphorylate and activate MEK1, although *in vivo* MEKK1 activation does not apparently result in increased ERK activity (Minden *et al.*, 1994). Ras is able to talk to the JNK cascade, possibly via its activation of the JNK KKK (PAK-1 in some systems). PAK (p21 associated kinases) are apparently effectors of the Rac GTPase (Manser *et al.*, 1994) but is is not clear which, if any, MAP kinase modules they regulate. This type of network is not yet fully elucidated, not least because information on the relative significance of inetractions between kinases is not available. Moreover, it is clear that the current picture contains data produced across different cell systems. Whilst these networks are challenging to understand experimentally they are clearly ingenious mechanisms which enable single inputs to govern multiple biochemical events.

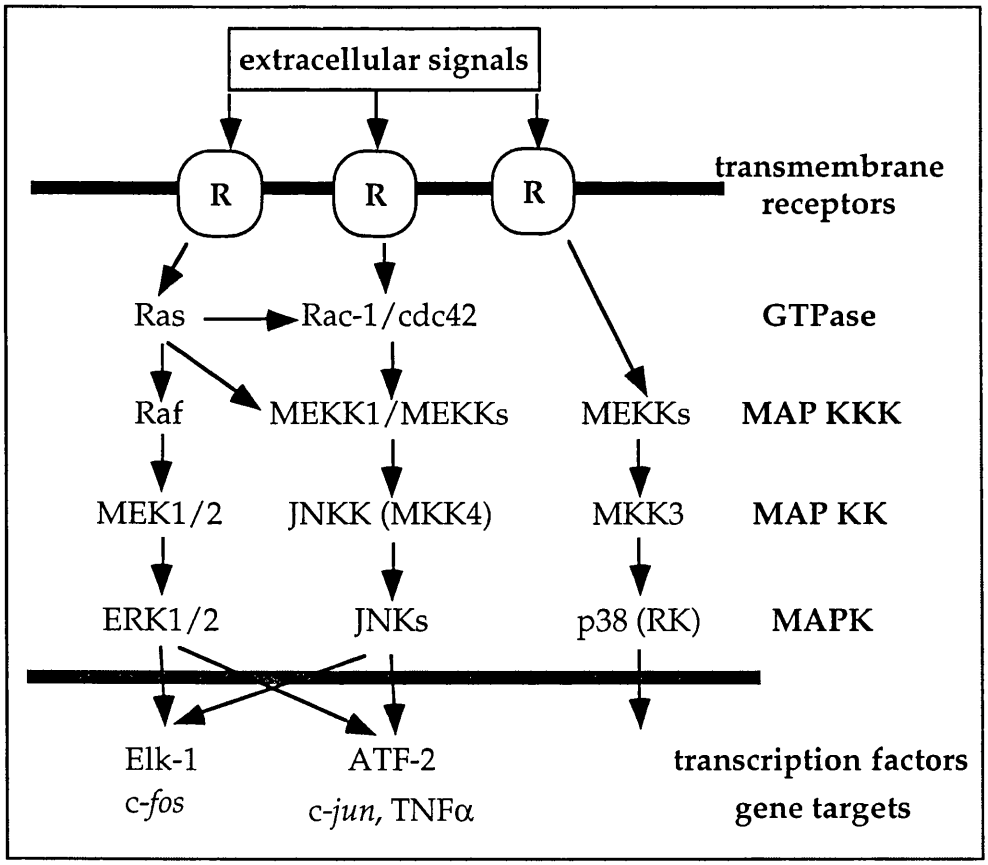


Figure 1.5. MAP kinase signalling modules and their nuclear targets. Crosstalk between cascades is extensive, for simplicity only defined *in vivo* interactiona are shown. Extracellular signals include mitogens, cellular stress and cytokines.

1.4.5 Modular design of signalling molecules within GTPase/kinase cascades imparts efficiency.

Signal transduction proteins have distinct domains which are involved in catalysis and protein-protein interaction, these are reviewed in (Cohen *et al.*, 1995; Pawson, 1995; Pawson and Gish, 1992). Signalling enzymes do not have to interact

with a large number of small substrates, it is now recognised that sophisticated signalling cascades are based on the highly specific transmission of a weakly amplified signal to target effector proteins. Thus, it is being recognised that signalling proteins are modular. They contain structural domains which allow for diversification within a single polypeptide. Typically individual domains may be responsible for 1) establishing a protein-protein interaction with upstream regulator, 2) positioning of substrate within active site and 3) targeting of the signalling protein to a specific subcellular location. Formation of these protein-protein interactions seems to be almost exclusively transient in mammalian signalling models. The formation of pathways seems to be activation dependent. There are very few examples of 'hard-wiring' in mammalian systems. Yeast models seem to provide more evidence of the pre-existence of protein super-complexes which are assembled ready for reaction to an input. However, as discussed above, these super-complexes may be merely unrecognised currently in mammalian systems.

A number of protein domains have been identified as important signalling modules. Their common properties are that they have compact structures which are retained when the domain is taken out of its protein context and they have closely spaced N- and C-termini. This latter point means that they can be visualised as simply plugging into the surface of a protein (Cohen *et al.*, 1995). Characterised modular binding domains are described below. Emphasis is placed on SH2 and SH3 domains since the FcεR1 regulation of an SH2/SH3 containing adapter molecule is analysed in Chapters 3 and 4.

1) *Src-homology 2 domains (SH2).* Protein phosphorylation upon tyrosine residues is a major mechanism for information transmission in mammalian cells. SH2 domains recognise phosphorylated tyrosine residues. Their affinity for phosphopeptide ligands is in the range of K_d=10-100nM. SH2 domains have essentially no affinity for unphosphorylated peptide sequences. SH2 domains are highly specific, despite the fact that their core target, phosphotyrosine, is present upon a large number of proteins within an activated cell. A single amino acid change in an SH2 domain is sufficient to redirect the specificity of a *src* SH2 domain towards targets of the *C. elegans* SEM-5 adapter molecule. Their specificity is apparently determined by the protein sequences which are C-terminal to the phosphotyrosine residue. Libraries of phosphopeptides have been used to examine specificity determination of SH2 domains (Songyang *et al.*, 1993). Phosphopeptides were synthesised with randomised sequences at positions Tyr+1 to Tyr+3 and SH2 domains were tested for their affinity as fusion proteins to extract a particular peptide from the library. On the basis of these data, the SH2 domains were divided into 2 classes. Class I SH2 exhibited a preference for +1, +2 hydrophilic and +3 hydrophobic. Class

II SH2 preferred +1 hydrophobic, +2 no preference, +3 hydrophilic (Songyang *et al.*, 1995). It should be noted that SH2 domains will bind proteins other than their single high affinity target with significant affinity. Therefore, the availability of a particular target within a cellular context may be an important determinant of their biological effects. Experimentally this has another implication. Driving of an SH2 domain-target equilibrium towards binding, for example by provision of high local concentrations of SH2, will lead to non-physiological interactions being detected as positive.

The result of a productive interaction between an SH2 domain and a phosphotyrosine residue is that a signal is transmitted Mechanisms for this are as follows; *a) Recruitment of a substrate into the subcellular context of its regulator enzyme.* A prototypical example of this is in the juxtaposition of the Sos GEF with its membane bound target GTPase, Ras. As will be discussed in detail later, SH2 domains of the adapter molecule Grb2 interact with phosphorylated tyrosines in the environment of a transmembrane receptor. Grb2 binds constitutively to Sos, and its activation dependent relocation to the plasma membrane brings Sos into the context of Ras (Buday and Downward, 1993; Chardin *et al.*, 1993; Downward, 1996). *b) Alteration in the catalytic activity of SH2 containing proteins or their binding partners.* Binding to a kinase via an SH2 domain may result in phosphorylation of the SH2 domain containing protein. This can result in an alteration in catalytic activity or can create a novel binding site for further SH2 mediated protein-protein interactions.

2) *Src-homology 3 domains (SH3).* SH3 binding sites are composed of Proline rich decapeptides. These peptides bind to SH3 domains with K_d=5-100μM. Specificity is apparently conferred by interaction of non-Proline residues in the ligand and variable loop regions in the SH3 domain. As for SH2 interactions, mutagenesis studies have identified a range of binding preferences for SH3 domains. The consensus core site is X-Pro-Pro-X-Pro, where X are hydrophobic. Covalent modifications such as phosphorylation of target proteins are not required for SH3 docking. However, there is evidence that activity of some SH3 binding proteins may be feedback downregulated by phosphorylation. Current models are based on the concept that SH3 binding results in conformational change in the target protein which permits the initation of downstream signalling events. The result of productive interactions between SH3 domains and ligand are varied:

a) SH3 domains may mediate localisation of a target protein to a specific cellular compartment.. SH3 domains mediate protein localisation to the plasma membrane or cytoskeleton. Bar-Sagi *et al* show that SH3 domains of PLC-γ1 and Grb2 target those proteins to cytoskeletal microfilaments and membrane ruffles respectively (Bar-Sagi *et al.*, 1993). This targeting is independent of the SH2 and other domains present in the two molecules. Concomitant with targeting to a cellular compartment, SH3 domain

mediated interactions may bring enzymes into proximity with their substrates. *b) SH3 domains can regulate enzymatic activity in a cis- or trans-fashion.* The SH3 domain of Abl negatively regulates Abl tyrosine kinase activity (Mayer *et al.*, 1993). Mutations in the SH3 domain can alleviate this inhibition. Moreover, the GTPase activity of Dynamin is regulated by the docking of SH3 domains derived from a range of adaptor molecules and kinases including Grb2, Src and Fyn (Gout *et al.*, 1993).

3) *Alternative protein modules.* The SH2 and SH3 domain mediated interactions described above are not the sole mechanisms by which phosphotyrosine or Proline rich sequences are detected. Phosphotyrosine binding (PTB) domains are an alternative mode for interaction with tyrosine phosphorylated proteins (Van der Geer and Pawson, 1995). The PTB domain is not fully characterised but apparently its recognition is determined by sequences N-terminal to the Tyrosine. The prototypical exapmple of the PTB domain has been described for in the Shc adapter molecule. The WW domain (Sudol, 1996) has specificity for a variant of the Pro rich region, where two Proline residues are separated by one aliphatic residue from a phosphorylatable tyrosine. Finally, Pleckstrin homology (PH) domains are found to interact with both proteins and inositol phospholipids (Harlan *et al.*, 1994; Ingley and Hemmings, 1994). In this latter respect they may be an important connection between the protein and lipid signalling networks and may play critical roles in recruitment of proteins to lipid rich regions of the cell.

1.5 Regulation and function of Ras family GTPases.

1.5.1 Four classes of switch regulator control the guanine nucleotide binding status of Ras family GTPases.

The guanine nucleotide binding status of Ras family GTPases is tightly controlled. Four classes of switch regulator protein have been described which are used to regulate GTPase activation. These enzymes are described briefly below and are reviewed in (Boguski and McCormick, 1993).

1) *Guanine Nucleotide Exchange Factors (GEFs).* GEFs mediate the exchange of GDP for GTP and hence conversion to the activated state. GEFs associate with the GDP-bound GTPase, causing an increase in the rate of GDP shedding. Detectable stable complexes of GEF and empty GTPase are formed, although they will persist for only a short time physiologically. On binding of GTP, the GEF dissociates. GDP binding will also dissociate the GEF at this point. It seems likely therefore that there is a level of regulation of GEFs which impart a preference for complex formation with the GDP-bound GTPase.

2) *GTPase activating proteins (GAPs).* GTP is slowly converted to GDP by the intrinsic hydrolytic capability of the GTPase. GAPs accelerate this process by up to 20 fold. There are two current models on the consequences of GAP binding to GTPases, both based on data from the Ras GTPase/p120 Ras-GAP system. p120 Ras-GAP undergoes a conformational change on interaction with Ras. One model is that this change allows GAP to interact with other proteins and act as a true, information transmitting, Ras effector. Alternatively, interactions with upstream regulatory proteins affect the enzymatic activity of p120 Ras-GAP and hence modulate Ras status. Here, p120 Ras-GAP is a mechanism by which other signalling pathways may gain access to Ras.

3) *Guanine nucleotide dissociation inhibitors (GDIs).* GDIs inhibit the dissociation of GDP from GTPases. In addition, GDIs can recognise the GTP bound forms of some GTPases and protect them from the action of GAPs. In the case of the Rab GTPases a further consequence of GDI activity has been observed. The RabGDI molecule binds Rab3A.GDP and prevents exchange of GTP for GDP. Moreover, this interaction also prevents Rab3A.GDP from becoming membrane localised. By analogy with other Ras family members, membrane localisation is required for interaction with primary effector molecules.

4) *Prenyltransferases (PTs).* Access of Ras family GTPases to their effector proteins is apparently dependent upon their being anchored to various cellular membranes. Hence Ras proteins are prenylated at their C-termini with the covalent attachment of farnesyl or geranylgeranyl groups. The enzymes responsible for these post-translational modifications are clearly important functional GTPase regulators. However, very little is currently understood concerning their regulation.

1.5.2 Roles of Ras family GTPases

Ras proteins are primarily involved in governing cellular processes that lead to growth and differentiation. Some 40% of human cancers have been shown to contain activating mutations in Ras proteins. These mutations tend to generate a constitutively GTP loaded Ras protein which may be resistant to GAP activity. The net result of this is a dysregulation of Ras signalling pathways which directly influence growth and differentiation. By definition, growth and differentiative programmes require the induction of multiple genes. Ras is thus able to mediate its functions by control of multiple signalling pathways of the type described above, where MAP kinase cascades regulate diverse transcription factor targets.

GTPases of the Rho subfamily (Rho, Rac and CDC42) have also been shown to control the activation of transcription factors. These GTPases activate MAP kinase cascades in a manner analogous to Ras. There is also significant cross-talk between signalling cascades initiated by the different GTPases. There is an extensive literature on Rho-family GTPase control of the actin cytoskeleton. Growth factors which regulate cell growth and differentiation cause profound alterations in cellular morphology. This morphological change is dependent upon rearrangements of the actin cytoskeleton. Rho and Rac and CDC42 are apparently essential components of pathways which control reorganisation of actin in a growth factor dependent fashion (Tapon and Hall, 1997). Upon receipt of a growth factor signal, actin is re-polymerised into defined structures. These events have been best studied in the Swiss 3T3 fibroblast system. In Swiss 3T3, Rac-1 directs the formation of membrane ruffles and lamellopodia (Ridley *et al.*, 1992). RhoA has a distinct function in governing the formation of focal complexes and stress fibres (Ridley and Hall, 1992). The former structures are rich concentrations of intercellular adhesion molecules and intracellular signalling proteins which are important interfaces between cell and environment. Activation of CDC42 also has a profound effect upon cellular morphology, causing formation of microspikes or filopodia (Nobes and Hall, 1995). It seems likely that membrane ruffle and filopodia formation are aspects of a similar response to cellular stimulation; the generation of increased contact area with the environment.

1.5.3 Features of Ras family GTPases.

Ras family GTPases are regulatory molecules which govern functional outcomes to multiple cellular input. Information received by GTPases may derive from growth factor receptors, antigen receptors and alterations in the extra- or intra-cellular environment. There are certain features of GTPases which contribute to the sensitivity and efficiency of the signalling pathways which they control. These include the ability of one GTPase to regulate the activity of another and the multiple downstream effector pathways which any one GTPase can regulate.

1) *GTPases act in cascades.*

GTPases can act in a networked fashion, there is much evidence that the activity of one GTPase can affect that of another. In pathways for the organisation of the actin cytoskeleton in the Swiss 3T3 system, CDC42 leads to activation of Rac-1 and then Rho (Nobes and Hall, 1995; Tapon and Hall, 1997). In oncogenic transformation of fibroblasts, there is a critical role for Rac-1 in Ras mediated transformation (Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995). In this system, Rac and Ras are strongly transforming in both Rat-1 and NIH3T3 fibroblasts, whilst Rho is only weakly transforming in NIH3T3. Rac-1 and Rho are both essential for Ras transformation However, in assays for cytoskeletal rearrangement and transformation

is is clear that Ras pathways bifurcate to Raf-1/MEk and Rac-1 dependent signalling cascades. Inhibition of Rac-1 function affects Ras but not Raf-1 mediated transformation. Moreover, active Raf-1, Rac and Rho synergise to mediate transformation, suggesting that they are not on a linear pathway.

2) *GTPases have multiple effector molecules.*

All genes for Ras proteins encode a nine amino acid sequence in the amino-terminal region of the protein which undergoes a major conformational shift when Ras binds to GTP (Ma *et al.*, 1995; Marshall, 1996). This 'effector loop' region can be mutated without altering the guanine nucleotide binding status of the GTPase but with profound effects on the effector molecules which bind to the GTPase. Hence this region is a determinant of the effector pathway selected by the GTPase from the complement of biochemical effector molecules in its environment in response to a particular stimulus. Recent data for Ras and Rac proteins have defined effector loop point mutations which are selective for a particular effector and exclusive of others (Dieckmann *et al.*, 1995; Lamarche *et al.*, 1997; Tapon and Hall, 1997; Westwick *et al.*, 1997). The use of these effector loop mutants has begun to assign functions to particular GTPase effector molecules.

The complement of characterised Ras effector proteins is summarised in Chapter 4. It is clear that both Ras and Rho family GTPases regulate a broad spectrum of structurally unrelated effector proteins. Protein kinases, lipid kinases, regulators of GTPase activity and proteins containing transcription factor motifs are all represented in the literature as potential effectors of GTPase signalling. Some, but not all, effectors of Rac/CDC42 have been shown to contain a consensus sequence motif which apparently confers the ability to interact with the GTPase. The CDC42/Rac interactive binding (CRIB) motif (Burbelo *et al.*, 1995) is an 18 amino-acid sequence found in the p21 associated Ser/Thr kinase (PAK) (Manser *et al.*, 1995; Manser *et al.*, 1994), the tyrosine kinase p120-ACK and the Wiskott-Aldrich Syndrome Protein (WASP) (Symons *et al.*, 1996). CRIB motifs are not absolutely required for a protein to act as a Rac/CDC42 effector. For example, the Partner of Rac-1 (POR1) (Van Aelst *et al.*, 1996), p67 phagocyte oxidase (Diekmann *et al.*, 1994) and PI (4)P 5-kinase molecules (Hartwig *et al.*, 1995) are CDC42 or Rac-1 effectors which do not contain CRIB motifs.

1.6 GTPase/kinase cascades in antigen receptor signalling.

Ligation of antigen receptors on cells of the immune system results in profound changes in cell fate. Antigen receptor regulated responses are numerous, including 1) decisions concerning growth, survival, differentiation and 2) expression of effector function. Within this latter class of events falls the production of soluble mediators

such as cytokines and growth factors, the secretion of effector proteins such as cytotoxic enzymes and immunoglobulins and the surface expression of molecules which are involved in the perception of the extracellular environment. Moreover, in response to antigen receptor ligation cells undergo morphological changes. These are specifically involved in the formation and stabilisation of cell-cell contacts, for example between an antigen presenting cell and effector lymphocyte, or between leukocyte and endothelium prior to tissue infiltration. Within this extensive complement of receptor coupled responses, it is likely that Ras family GTPases will play multiple roles in transduction of antigen receptor signals.

There has not been extensive examination of GTPase signalling in multiple facets of cellular activation in the immune system. Two lines of study suggest that antigen receptor regulation of GTPases may be of functional importance. First, it is a conserved feature of antigen receptor signalling that receptor ligation results in activation of guanine nucleotide exchange factors (GEFs) for Ras family GTPases. Second, studies in the developing and mature peripheral T lymphocyte have demonstrated important roles for at least one GTPase/kinase cascade. These studies are summarised below.

1.6.1 Antigen receptors regulate GEFs for Ras family GTPases.

Stimulation of Ras dependent signalling pathways is dependent upon GTP loading and hence activation of the initating Ras molecule. GTP loading of Ras is promoted by the Sos guanine nucleotide exchange factor. This is a conserved feature of early signalling in response to both B- and T-cell antigen receptors (Alberola-Ila *et al.*, 1997; Gold *et al.*, 1993; Smit *et al.*, 1994). The mechansim of Sos recruitment to the plasma membrane and subsequent interaction with Ras has been studied in some depth and is a focus of the experimental work presented in Chapter 3 of this thesis.

Firstly, Sos is constitutively bound through its Proline rich region to the SH3 domains of the Grb2 adapter molecule. Grb2 is recruited via its SH2 domains to Tyrosine residues in the receptor microenvironment which have been phosphoacceptors for receptor activated PTKs (Buday and Downward, 1993; Buday *et al.*, 1994; Reedijk *et al.*, 1990; Reif *et al.*, 1994). This places Sos in a position to interact with the membrane. A recent paper from the Bar-Sagi laboratory shows that the Sos PH domain regulates the inducible association of Sos with the plasma membrane (Chen *et al.*, 1997). Whilst Sos can bind PIP_2 via its PH domain, this is apparently an interaction which sequesters Sos away from interaction with Ras. On serum stimulation Sos relocates to the plasma membrane in a PIP_2 independent manner, overexpression of Sos PH domains acts in a dominant negative fashion on stimulation of the Ras/Raf-1/MEK. Juxtaposition of Sos and Ras results in exchange of GTP for GDP. Membrane targetting of Sos by fusion with a myristoylation site

results in the activation-independent GTP loading of Ras and hence constitutive activation of downstream signalling pathways (Aronheim *et al.*, 1994).

Lymphoid and mast cells express Vav-1, a multidomain signalling protein which is tyrosine phosphorylated in response to TCR and FcεR1 ligation (Katzav *et al.*, 1994; Margolis *et al.*, 1992). Vav contains multiple protein modules including SH2 and SH3 domains involved in protein-protein interaction, a dbl homology domain encoding GEF function and a PH domain thought to bind phosphorylated inositol lipid species. It has recently been shown that Vav-1 does in fact act as a GEF for Rac-1 and CDC42 (Crespo *et al.*, 1997). Vav-1 apparently has no exchange activity for Ras. Vav-1 interacts with a component of the Grb2 adapter molecule complex in T cells, the SLP-76 protein (Tuosto *et al.*, 1996). The functional consequences of this interaction for T cell activation have been partly explored. Overexpression of Vav-1 in T cells drives the induction of the Nuclear Factor of Activated T cells (NFAT), a transcription factor which is important in control of IL-2 gene induction (Wu *et al.*, 1996). Another functional consequence of Vav-1 activation, shown in Cos cells heterologously expressing the FcεR1, is induction of Jun kinase activity (Teramoto *et al.*, 1997). This presumably reflects Vav-1 dependent activation of a Rac-1 GTPase kinase cascade where the terminal effector kinase is JNK (Crespo *et al.*, 1996).

The role of Vav-1 *in vivo* has been evaluated using Vav-1 deficient (Vav-1 -/-) mice. B and T cells from these mice show defective proliferative responses to antigen (Fischer *et al.*, 1995; Tarakhovsky *et al.*, 1995). A mast cell phenotype for Vav-1 -/- animals has been less straightforward to assign. All functional assays for mast cell activation in Vav-1 -/- mice apparently match those performed in normal littermate controls. Vav-1 -/- mast cells are not defective in FcεR1 driven PTK activation, ERK or Jun kinase activation or secretory responses. The status of FcεR1 induced calcium flux has not been assayed in Vav-1 -/- mast cells. The simplest explanation for these data is that Vav-1 is not required for mast cell activatioon. However, unlike T and B cells, mast cells express Vav-2, which may compensate in part or wholly for the Vav-1 deficiency. Studies with a double Vav-1/Vav-2 knockout are proposed to address this issue. Mast cell studies using Vav-1 -/- mice are not published and are a personal communication from Dr Victor Tybuliewicz, National Institute for Medical Research, London.

1.6.2 Activation of the Raf-1/MEK/ERK cascade is important in T cell development and peripheral T cell function.

The only GTPase/kinase cascade to be intensively studied in the context of an antigen receptor stimulated cell is the Ras/Raf-1/MEK/ERK cascade in the context of the T lymphocyte. The laboratory of Roger Perlmutter has created transgenic models for the role of the Ras/Raf-1/MEK/ERK signalling cascade in thymocyte development

(Alberola-Ila *et al.*, 1997). The data show that thymocytes from mice expressing dominant inhibitory Ras do not undergo positive selection (i.e. do not progress to mature T cells, differentiating into CD4 and CD8 bearing populations). Expression of catalytically inactive MEK also blocks positive selection but leaves negative selection (by apoptosis) intact. Hence the Ras/Raf-1/MEK cascade has been shown, *in vivo,* to be a critical regulator of commitment of an antigen receptor stimulated cell to a particular differentiative programme.

The Ras/Raf-1/MEK/ERK cascade has also been experimentally manipulated in a model of mature peripheral T lymphocyte function. Clonal expansion of T cells in response to antigen is dependent upon induction of the genes for IL-2 and the IL-2 receptor (IL-2R). In an autocrine fashion IL-2 ligates IL-2R, signalling T cells to progress through the G1/S phase of the cell cycle and hence proliferate. Induction of transcription factors which control IL-2 promoter induction, such as NFAT, has been the object of intensive study. This is because the signalling pathway for NFAT induction is the target of the immunosuppressive drugs Cyclosporin A and FK506.

NFAT activity is dependent upon the Ras GTPase (Genot *et al.*, 1996; Woodrow *et al.*, 1993). At least two Ras effector pathways link Ras and the transcriptional activity of NFAT. Inhibition of the MEK kinase compromises TCR induction of NFAT, suggesting that the Raf-1/MEK cascade is required for this response. Moreover, a Ras dependent Rac-1 signal is also involved in TCR regulation of NFAT. The Raf-1/MEK cascade apparently targets multiple transcription factors in a mature T cell model system, since TCR induction of Elk-1 is also dependent upon this signalling pathway (Genot *et al.*, 1996). These data place a single Ras effector kinase cascade, the Raf-1/MEK/ERK cascade, upstream of the regulation of two distinct transcription factor targets in mature TCR stimulated T lymphocytes.

1.7 The potential for GTPase/kinase signalling in the FcεR1 system.

Antigenic crosslinking of the FcεR1 results in mast cell activation and expression of function. To date signalling via GTPase/kinase cascades in this system has not been studied. However, GTPase regulation may be an important consequence of antigenic crosslinking of the FcεR1. The FcεR1 may regulate of two characterised guanine nucleotide exchange factors for Ras family GTPases, Sos and Vav-1. There has been no analysis of the membrane proximal signalling events characterised for other antigen receptor systems which may link the FcεR1 to GEFs such as Sos.

Several known functions of Ras family GTPases may contribute to mast cell activation in response to FcεR1 ligation. Activated mast cells undergo pronounced morphological changes, including the formation of focal complexes, stress fibres and membrane ruffles (Barker *et al.*, 1995; Norman *et al.*, 1994; Pfeiffer and Oliver, 1994; Price *et al.*, 1995a). These are clearly candidate processes for the involvement of

GTPases such as Rac-1 and Rho. Moreover, FcεR1 ligation results in induction of the genes for multiple cytokines and chemokines, including the NFAT-regulated gene for IL-4 (Weiss *et al.*, 1996). FcεR1 stimulation also induces the synthesis of immediate early gene products such as the Fos (Stephan *et al.*, 1997) protein; Fos being critically controlled in fibroblasts by the transcription factor Elk-1, a target of the Ras GTPase (Price *et al.*, 1995b; Treisman, 1994).

1.7.1 SUMMARY AND AIMS

This introductory section has established that an understanding of the mechanisms by which the FcεR1 controls mast cell function is an important aim in immunology. In comparison with other antigen receptors, signal transduction by the FcεR1 is poorly understood. Moreover, whilst the mechanisms and targets for transcriptional activation are understood in general terms, for example the use of GTPase/kinase cascades to regulate transcription factors, these concepts have not been applied to the FcεR1 system. The aims of the work presented in this thesis were therefore as follows:

1) Identification of upstream regulatory signalling cassettes for GTPase activation in FcεR1 activated cells.

2) Identification of transcription factor targets for GTPase/kinase cascades in FcεR1 regulated cells and characterisation of the relevant effector pathways.

1.7.2 Choice of model system.

Primary mast cells are difficult to isolate in significant numbers. Lung tissue from individuals who were asthmatic/atopic will carry large numbers of bronchial mast cells, but these cells will be primed rather than naive and thus do not represent a good model for the study of responses to antigenic stimulation. There are reliable methods for the isolation of bone marrow-derived mast cells (BMMC), which involves the three week culture of bone marrow isolate in IL-3, by which time the population will be >90% mast cells. However, like the primary cultures, BMMC are 1) subject to variation according to the individual from whom they were isolated, 2) not suitable for transient transfection and 3) require the continual presence of a second stimulus (i.e. IL-3) during the study of FcεR1 signalling.

There are a number of immortalised mast cell lines available which overocome the problems noted above. The RBL2H3 cell line is derived from a rat basophil leukaemia and displays phenotypic characteristics of mucosal-derived mast cells. RBL2H3 have high levels of FcεR1 expression and may be readily transfected.

RBL2H3 are a variant of the original RBL line which is selected for a granular appearance and reasonable secretory response to IgE/antigen. RBL2H3 are an adherent line and thus partially model a case where IgE/antigen responses occur in the context of adherence to a tissue substratum. RBL2H3 are not without many of the problems associated with the use of immortalised cell lines. However, they represent a readily manipulable system for the study of FcεR1 signal transduction.

CHAPTER TWO

Materials and Methods

2.1. Reagents.

Sources of reagents were as follows: Recombinant mouse immunoglobulin E, raised against di-nitro phenol (IgE anti-DNP) was from Sigma. Keyhole Limpet Hemocyanin, conjugated to di-nitro phenol (DNP) was from Calbiochem. Ionomycin (Ca^{2+} salt), phorbol-12,13-dibutyrate (PdBu) and wortmannin were also from Calbiochem. The MEK kinase inhibitor PD098059 was from New England Biolabs. The Ro-318429 PKC inhibitor was a generous gift from Dr David Williams (Roche, UK). [^{32}P]γ-Adenosine tri-phosphate (ATP) and [^{14}C] acetyl coenzyme-A were from Amersham. Non-isotopic acetyl coenzyme-A, and chloramphenicol were from Sigma. Nucleotides (ATP, CTP, GTP) were from Calbiochem. Prestained molecular weight protein markers were from Gibco (UK). Rhodamine-Phalloidin was from Sigma. Protogel solution was from National Diagnostics. Streptolysin O was from Wellcome.

2.2. Antibodies.

The following monoclonal antibodies were purified from hybridoma supernatants using Protein A affinity chromatography, and were supplied by the ICRF Hybridoma Development Unit: UCHT-1 raised against human CD3ε subunit (Beverley and Callard, 1981), 9E10 raised against the myc epitope (Evan *et al.*, 1985), OX34 raised against rat CD2 (He *et al.*, 1988) and the JRK1 anti-FcεR1β was a generous gift of Dr. Juan Rivera, NIH, Bethesda, MD. Anti-GFP Western analysis was performed using an affinity purified rabbit anti-GFP, which was a generous gift of Dr Ken Sawin, Imperial Cancer Research Fund.

Commercial sources of antibodies were as follows: monoclonal 4G10 against phosphotyrosine, polyclonal mSos1 against Sos and monoclonal anti-Shc, were from Upstate Biotechnology Inc. The monoclonal antibodies against Grb2, and epitopes common to p42 and p44 MAP kinase (pan-MAPK), and polyclonal anti-Raf-1 were purchased from Affiniti. The pan-Ras (OP41) monoclonal was from Oncogene Science. Concentration of primary antibodies used in Western analyses are indicated in figure legends.

2.3. Peptides.

The following peptides were synthesised by Nicola O'Reilly and Elisabeth Li at the ICRF Oligopeptide Synthesis Service. Phosphorylated tyrosine residues are represented by pY.

Peptide	Sequence
EGFR-Y(1068)-P	PVPEpYINQS
Sos-Pro	SKGTDEVPVPPPVPPRR (Bowtell *et al.*, 1992)
Trk-Y(490)	IENPQYFSDA

2.4 Desalting of peptides.

It was necessary to desalt peptide stocks before coupling and use as affinity matrices. Desalting was carried out on Sephadex G-15 columns (Pharmacia) as follows. Sephadex gel (20g) was preswollen overnight at room temperature in PBS/0.02% NaN_3 (20ml). Biorad columns were packed with 10ml Sephadex G15 bed volume per 50mg peptide to be desalted. The packed column was washed once with NH_4HCO_3 (25ml 0.1M). Peptide was warmed to RT and then weighed in 10mg aliquots. Peptide was dissolved in NH_4HCO_3 (2.5ml 0.1M) and layered on to the top of the column. Three sequential fractions of 3ml were collected and snap frozen on dry ice. Frozen fractions were lyophilised, and then resuspended in dH_2O (2.5ml). The lyophilisation was repeated, and the final powder stock was stored at -20°C.

2.5 Coupling of peptides to Affigel-10 affinity matrix.

For use as affinity matrices, peptides were coupled to Affigel 10 activated ester agarose as follows. Affigel 10 was washed three times as a 50% solution in PBS. Peptide (1mg) was diluted to 1mg/ml in K_2HPO_4/KH_2PO_4 (pH 7.5, 0.5M), and incubated with washed Affigel 10 at 5mg/ml packed beads for 2h at 4°C. Uncoupled sites were blocked with (0.2M) ethanolamine for 1h at 4°C. Beads were washed three times in cold PBSA and stored at 4°C in 0.02% NaN_3/PBS. Control beads were produced with blocked sites by incubation with ethanolamine (0.2M) for 1h at 4°C, followed by washing and storage as above.

2.6 Glutathione S-Transferase Fusion Proteins.

Plasmids encoding the following Glutathione S-Transferase (GST) fusion proteins were a gift of the Signal Transduction Laboratory, ICRF and were described in (Egan *et al.*, 1993).

Fusion	Molecule
GST	GST alone
GST-Grb2	Full length human Grb2
GST-Grb2NSH2	N-terminal SH2 domain of Grb2 (aa 1-58)
GST-Grb2CSH2	C-terminal SH2 of Grb2 (aa 159-217)
GST-Grb2μSH3	Grb2 mutant 49L/203R
GST-Sos	aa 1135-1336 of mouse Sos 1

2.7 Preparation of Glutathione S-Transferase Fusion Proteins.

Fusion proteins were prepared as follows. *E. coli,* bearing fusion proteins encoded on the pGEX2T plasmid were inoculated into 400ml L-Broth containing Ampicillin (50mg/ml). Bacteria were grown to an OD_{550} of 0.6-0.8 before induction of fusion protein expression for 4h using isopropyl β-D thio-galactopyranoside (IPTG) (0.5mM) at 37°C. Cultures were then centrifuged, and resuspended at 4°C in PBSA (10ml) containing 1% Triton X-100, EDTA (2mM) and PMSF (1mM). Lysis was by sonication (3 x 1min, 50% power, on ice with 1 min recovery on ice between pulses) and cell debris was pelleted by centrifugation at 10000rpm for 10 min at 4°C. Initial purification of the fusion protein from lysate was by rolling incubation (2h) with 1ml of a 50% (v/v) solution of Glutathione Agarose beads which had been washed three times in cold PBSA containing 2mM EDTA. The suspension was then centrifuged at 3000 rpm for 30 seconds, washed twice in three volumes of the above lysis buffer, and twice in three volumes of PBSA/2mM EDTA.

The protein was eluted from the beads using glutathione (25mM) in Tris buffer (pH 8.0) on a BioRad 10ml column. The OD at 280nm was measured for each fraction. The protein fractions collected after elution were dialysed in Visking tubing (Mr cut-off 5000-8000 Da) against PBSA/2mM EDTA for 12h with three changes of buffer. Finally, fusion proteins were recoupled to glutathione agarose beads as a 50% solution by rolling incubation for 2h at 4°C. Coupled fusion protein beads were washed three times in three volumes PBS stored in 50% glycerol, 50mM Hepes (pH 7.4) and 50mM NaCl at -20°C.

2.8 Expression Plasmids used in transient transfection.

The following plasmids encoding active mutants of GTPases were used: pEF Ha-v-ras (S12V, A59T) (Izquierdo *et al.*, 1993), pEF V14RhoA-myc, pEF V12Rac-1myc (Hill *et al.*, 1995a). Dominant inhibitory GTPase mutants were as follows; RSV N17 Ras (Izquierdo *et al.*, 1993), pEF N17 Rac-1 and pEF N19 RhoA (Hill *et al.*, 1995a).. pEF dominant negative (DN) Raf-1 (Raf-1 amino acids 1-257) has been previously described (Turner and Cantrell, 1997). pEF Raf-1 CAAX was a gift from Dr Julian Downward, ICRF. The activated mutant of Calcineurin (CNM in the pSRα vector) has been described previously (Woodrow *et al.*, 1993) pEF C3 transferase, pRK L61 Rac-1, pRK L61Y40C Rac-1 and pRK L61F37A Rac-1 were gifts of Dr Alan Hall (University College London) (Diekmann *et al* ,1996; Lamarche *et al* ,1997).

2.9 Reporter constructs used in transient transfection.

The following reporter constructs were used in Chloramphenicol acetyl transferase assays.

pEF Nlex Elk-1 and pEFlexOPtk.CAT (Marais *et al.*, 1993); were a gift from Dr R. Treisman (ICRF).

AP1.CAT consisted of three copies of the AP1 binding site from the IL-2 promotor/enhancer linked to the CAT gene (Williams *et al.*, 1992).

IL-2 NFAT CAT comprised three copies of the NFAT/AP-1 site derived from the human IL-2 promoter (Woodrow *et al.*, 1993). IL-4 NFAT CAT comprised a trimerised NFAT/AP-1 site derived from Purine box B of the murine IL-4 promoter (Chuvpilo *et al.*, 1993). The oligonucleotide NFAT/AP-1 binding sites used in these constructs are shown below:

hIL-2 5' TAAGGAGGAAAAACTGTTTCATACAGAAGGCG 3'

mIL-4 5' GATCCTGAGTTTACATTGGAAAATTTTATAGAGCGAGTTG 3'

IL-4 (-270) CAT comprised a 270 bp fragment of the murine IL-4 promoter immediately 5' to the TATA box, fused to the CAT reporter gene (Chuvpilo *et al.*, 1993; Pfeuffer *et al.*, 1994).

Reporter genes for NFAT-GFP translocation assays were generated as follows by Dr Edward McKenzie, Yamanouchi Research Institute, Oxford. The full-length NFATc1 cDNA or a fragment corresponding to amino acids 418-716 of NFATC1 were subcloned into the pEGFP-C1 vector (Clontech) to give NFATC1-GFP and NFATC1 ΔRel-GFP respectively. Constructs were verified by sequencing (Dr Edward

McKenzie). All constructs were purified by CsCl density gradient centrifugation prior to use in transfection.

2.10 CsCl purification of plasmid DNA for use in transient transfection assays.

CsCl purification of plasmid DNA was carried out as follows. Briefly, a 400 ml culture of *E. coli* carrying the desired plasmid was grown overnight in BHI (brain heart infusion) medium. Bacteria were pelleted (4000rpm, 4°C, 20 min) and resuspended in 40 ml solution I (50mM glucose, 25mM Tris pH 7.4, 10mM EDTA). Bacterial cells were lysed by adding 80 ml of solution II (1% SDS, 0.2M NaOH). Denatured proteins, chromosomal DNA and cellular debris were precipitated by adding 40 ml of solution III (5M potassium acetate pH 5.0). Precipitates were removed by centrifugation (4000rpm, 4°C, 20 mins). Supernatant DNA was precipitated using 90 ml isopropanol followed by centrifugation (4000rpm, 4°C, 20 mins). The DNA pellet was resuspended in 5 ml distilled water, to which ammonium acetate (5ml 7.5M) was added to precipitate RNA. Samples were centrifuged as above and supernatants were ethanol precipitated for 1hr at -20°C. After centrifugation (4000rpm, 4°C, 20 min), DNA pellets were resuspended in 8.5ml dH_2O and ethidium bromide (0.5 ml 10mg/ml). 8.8g CsCl was dissolved in this solution and the mixture was divided between two heat-sealable ultracentrifugation tubes and centrifuged in a vertical rotor (VTi 65.2, Beckman) at 65000 rpm for 4 h at 20°C. The plasmid DNA was removed and the ethidium bromide extracted with water saturated *n*-butanol. The DNA was precipitated with 2 to 3 volumes of ethanol and resuspended in dH_2O.

2.11 Cell culture

The Rat Basophilic Leukaemia cell line RBL2H3 was a generous gift of Dr. Juan Rivera (National Institute of Allergic, Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD, USA). This cell line is derived from leukaemic mucosal mast cells. RBL2H3 were maintained as monolayer cultures in Dulbecco's Modification of Eagle's medium (DMEM) containing 10% (v/v) Foetal calf serum which had been heat inactivated (HI-FCS) by incubation at 55°C for 60 min. Cell cultures were incubated at 37°C in a 5% CO_2 atmosphere. For passage or use in experiments, RBL2H3 were detached from the culture substrate using cell scrapers.

In certain experiments, T lymphocytes were used in comparison with RBL2H3. Primary T cells were prepared from human peripheral blood by the discontinuous Ficoll-Hypaque gradient centrifugation method described by Cantrell and Smith (Cantrell and Smith, 1984). The Jurkat T leukemia line JHM-1 (bearing the human

muscarinic acetylcholine receptor) was maintained in RPMI containing 10% HI-FCS. Passage stocks were kept in 1mg/ml G-418 to ensure stability of the HM-1 clone.

2.12 Cell stimulation and lysis for protein analysis by Western blot.

Reagents for antigenic crosslinking of the FcεR1 were prepared, and stored, as follows. IgE-anti DNP was diluted in sterile PBSA and stored in aliquots at -20°C. KLH-DNP conjugate (400 DNP groups per molecule) was dissolved at 5mg/ml in PBS/2mM $MgSO_4$. Solubilisation of KLH-DNP was not straightforward, the 5mg/ml solution was sonicated (3 x 1min, 50% power) and then stirred overnight in the dark at RT. Undissolved material was removed by centrifugation and the KLH-DNP solution was stored at -20°C in aliquots.

RBL2H3 cells were detached from the culture substrate using cell scrapers and either stimulated in suspension or replated at the required density for 30 min prior to priming. Cells were incubated with 1μg/ml IgE anti-DNP in DMEM/10% FCS for 1h prior to antigenic crosslinking of the bound IgE using KLH-DNP conjugate at the indicated concentrations.

Cells were pelleted in a microfuge and washed once in ice cold PBS to remove serum proteins. Unless otherwise stated, post nuclear lysates were prepared as follows; cells were lysed for 30 min with rotation in 0.5 ml of the following buffer per 10^7 cells; 50mM Hepes pH 7.4, 150mM NaCl, 20mM NaF, 10mM iodoacetamide, 1mM phenylmethylsulfonylchloride, 1mM sodium orthovanadate and 1% (v/v) Nonidet P-40 (Buffer I). After lysis, nuclei were pelleted by microfugation (14000rpm, 30 sec, RT) and the post-nuclear supernatant removed to a clean tube.

For total cell lysate preparation, including nuclear extraction, (Chapter 7) the following protocol was followed; cells were lysed for 30 mins in a buffer containing 20mM Hepes pH 7.9, 20% (v/v) glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 10mM NaF, 1mM DTT, 1mM PMSF, 1mM Na_2VO_4 and 1% NP-40 (Buffer II). Nuclear membranes and DNA were pelleted by centrifugation at 14000 rpm for 20 mins at 4°C.

2.13 Acetone precipitation of cellular protein from lysate and sample preparation.

Acetone precipitation was used to prepare samples of the total protein in a cellular fraction. Here, 0.5ml lysate was added to 0.7ml acetone, after mixing the tubes were placed at -20°C for 1hr. Precipitated proteins were pelleted in a microfuge, the pellets were dried and resuspended in 75μl hot reducing sample buffer (1% SDS, 62.5mM Tris pH 6.8, 10% glycerol and 5% 2-mercaptoethanol). Samples were boiled for 10 min before loading.

2.14 Affinity purification of cellular proteins using Glutathione Agarose, or Affigel-10 matrices.

For affinity purifications using peptide, or fusion protein matrices, lysates were pre-cleared with insoluble Protein A solution (30µl 10% (v/v)) for 20 min with rotation at RT. A second pre-clear using either 30µl 50% (v/v) Affigel-10 with blocked sites, or 10µl 50% (v/v) glutathione agarose beads was carried out according to the type of affinity purification intended. Affinity purifications were for 1.5hr at 4°C with rotation. Beads were pelleted in a microfuge, and then washed three times in fresh lysis buffer (Buffer I above). Finally beads were dried using a Hamilton syringe and resuspended in 75µl hot reducing sample buffer before boiling for 10 mins.

2.15 Immunoprecipitations.

For immunoprecipitations, lysates were prepared as above, using Buffer I. Lysates were pre-cleared with washed (x 4) Protein G-sepharose solution (30µl 10% (v/v)). Typically antibodies were precoupled to Protein G-sepharose by incubation at pH 7.4 for 2-4hr with rotation at 4°C, followed by three washes in PBS, and storage at 4°C in PBS/0.02% NaN_3. Immunoprecipitation was carried out for 2hr with rotation at 4°C. Amounts of immunoprecipitating antibody are indicated in figure legends. Beads were washed three times in fresh lysis buffer, and dried by removal of liquid using a Hamilton syringe. Beads were resuspended in 75µl hot reducing sample buffer before boiling for 10 min.

2.16 Resolution by SDS-PAGE and Western blotting.

Proteins were resolved by SDS-PAGE using Protogel (National Diagnostics) 30% acrylamide, 0.8% bis-acrylamide stock solutions. The percentage acrylamide resolving gels (pH 8.8) used is indicated in figure legends. The stacking gels were 5% acrylamide (pH 6.8). For resolution of ERK kinase shift, a resolving gel containing 15% acrylamide/0.075% bis-acrylamide was used (Chapter 4). The running buffer used, contained 25mM Tris pH8.3, 190mM glycine, 3.5mM SDS. For NFAT-GFP mobility shifts (Chapter 7) proteins were resolved by 6% SDS-PAGE.

Resolved proteins were transferred to polyvinylidene difluoride (PVDF) membrane by electroblotting in a transfer buffer containing 10mM CAPS (pH11). The presence of protein on the membrane was checked by exposure for 30 sec to 2% Ponceau S stain and immediate de-stain by rinsing in H_2O.

Membranes were blocked by incubation in non-fat milk solution (5% w/v in PBS) for 1hr at RT. Antibody concentrations varied and are described in figure

legends. Typically primary antibodies were diluted in PBS/0.02% sodium azide/0.5% non-fat milk. Developing antibodies were horseradish peroxidase conjugated goat anti-rabbit, or donkey anti-mouse IgG diluted in PBS/0.05% Tween-20/0.5% non-fat milk. Washes between antibody incubations were standardised at four washes of 5 mins in approximately 150 ml PBS/0.05% Tween-20.

Where required, PVDF membranes were stripped of antibodies by incubation in a rolling hybridisation oven for 30 min in 25ml strip buffer (100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris pH 6.7) at 55°C. The membrane was rinsed three times in 50ml PBS/0.02% Tween-20 before blocking for 1h in 5% non-fat milk and re-probing at RT.

2.17 Streptolysin-O permeabilisation of RBL2H3 cells.

Streptolysin O (SLO) was dissolved in sterile PBS at 12 i.u./ml, and stored in aliquots at -70°C. Prior to use SLO was thawed on ice. A start solution was prepared containing 500μM MgATP, 500μM CTP, 2.0 i.u/ml SLO in an intracellular free calcium buffer designed to give 150nM $[Ca^{2+}]i$ in permeabilised cells. The content of this buffer was calculated using the 'Chelate' program. The 150nM $[Ca^{2+}]i$ buffer used, contained 150nM Ca, 3mM $MgCl_2$, 12.5mM PIPES, 12.5mM EGTA. Calcium buffer was corrected to pH 7.4 using a known volume of KOH and KCl was added taking into account the contribution of K^+ ions from the KOH to give a final $[K^+]$ of 150mM. The start solution was warmed to 37°C. Cells were washed once in warm PBSA, and resuspended in warm calcium buffer (800μl) , to which 200μl start solution was added. Thus final concentrations of nucleotides were 100μM MgATP, 100μM CTP and 0.4i.u/ml SLO. The cells were mixed gently, and stimuli were applied as for intact cells. Stimulations were stopped, and cells were lysed as for normal Western blotting protocols.

2.18 Transient transfection and CAT assays.

Transient transfection was carried out using a Beckman Gene-Pulser electroporation apparatus. RBL2H3 monolayer cells were detached from the culture flask using a cell scraper and resuspended at 2 x 10^7 cells per 0.6ml DMEM + 10% FCS at 37°C. Cells were pulsed in 0.4cm cuvettes (Beckman, UK) at 310V, 960μF before pooling, dilution in complete medium and division at 1ml/well between wells of a 24 well tissue culture plate. Cells were allowed to recover for 6h at 37°C before priming and stimulation as indicated.

For chloramphenicol acetyl transferase (CAT) reporter gene assays, 5x10^6 cells were lysed in 150μl of a buffer containing 0.65% (v/v) NP-40, 10mM Tris pH 8.0,

1mM EDTA and 150mM NaCl for 15 mins on ice. Lysates were then transferred to a 68°C water bath for 10 minutes. Cell debris was pelleted and aliquots of lysate were removed to a fresh tube in an assay volume of 100µl, to which 40µl of a start solution containing 0.5mM acetyl CoA, 5mM chloramphenicol, 0.5M Tris pH 8.0 and 1µl/point of 50µCi/ml ^{14}C acetyl coenzyme A was added. The assay was incubated for 16h at 37°C before chloramphenicol was extracted using 150µl per point ethyl acetate. The amount of radioactivity in the acetylated product (100µl top phase) and non-acetylated substrate (50µl bottom phase) for each reaction was determined by liquid scintillation counting of organic, and aqueous phases, respectively. Results are expressed as percentage conversion of chloramphenicol to the acetylated form.

2.19 Cell imaging and NFATC1-GFP translocation assays.

RBL2H3 cells were cultured as described previously and electroporated using a Beckman-Gene Pulser apparatus at 10^7 cells/0.5ml DMEM, 960µF, 310V. After plating onto glass coverslips, cells were allowed 6hr recovery. Cell stimulation was carried out by 1h incubation at 37°C with 1µg/ml IgE anti-DNP (Sigma) followed by antigenic crosslinking of the FcεR1 using 250ng/ml (KLH)-DNP (Calbiochem) unless otherwise indicated. Cells were fixed in 4% paraformaldehyde for 30 min at room temperature. Nuclear staining using PI was carried out by permeabilisation of cells in 0.1% Triton-X100 (4 min, at RT) followed by sequential incubation with 500mg/ml RNase (37°C, 10 mins) and 0.1µg/ml PI (RT, 10 mins). Coverslips were washed in PBS after each stage.

The coverslips were mounted in 15µl Gelvatol which was prepared as follows. 2.4g Gelvatol (Monsanto Chemicals) was added to 6ml glycerol and left for 2hr at RT. To this mixture 12ml 0.2M Tris pH 8.0 were added and the solution was heated to 50°C for 10 mins with stirring. The resulting solution was clarified by centrifugation and aliquoted for storage at -20°C.

Gelvatol fixative was allowed to set for 4hr before imaging using a Leica TCS-NT upright confocal microscope. Images were corrected for contribution of PI emission to green fluorescent protein (GFP) signal and viewed using the Imaris software. In scoring experiments cells were analysed using a Nikon Axiophot inverted fluorescence microscope. Cells were scored for NFATC1-GFP localisation as blind-coded samples.

2.20 Rhodamine-Phalloidin staining for polymerised actin.

RBL2H3 cells were seeded onto glass coverslips and stimulated in complete medium. Coverslips were rinsed once to remove serum proteins and fixed in 4% paraformaldehyde before permeabilisation of cells in 0.1% Triton-X100 (4 min, at RT) and three washes in PBS. Coverslips were incubated with 0.25µg/ml Rhodamine-Phalloidin (Sigma) in PBS for 40 mins at RT in the dark. Coverslips were washed three times in PBS before mounting in Citifluor (Molecular Probes Inc.) and analysis by confocal microscopy.

CHAPTER THREE

Regulation of the adapter molecule Grb2 by the FcεR1 in mast cells.

3.1 INTRODUCTION

The data presented in this chapter will detail studies into membrane proximal signalling events which follow antigenic crosslinking of the FcεR1. The activation of receptors with associated or intrinsic tyrosine kinase activity induces the assembly of signal recognition complexes, initiation of intracellular signalling cascades and regulation of cellular responses. Antigen receptors regulate Ras and Rho family GTPases. These GTP binding proteins are central to the regulation of diverse cellular responses, including actin cytoskeleton re-arrangements and induction of transcription factor activity.

As reviewed in Chapter 1, GTPases cycle between GTP-bound (active) and GDP bound (inactive) states. Their GTP binding status is tightly regulated by several classes of protein. It is clear that two main mechanisms are employed by receptors to regulate the rate of GTP/GDP exchange and hence activation status of a GTPase: 1) an adapter molecule is used to recruit a guanine nucleotide exchange factor (GEF) to the membrane and hence into proximity with the prenylated GTPase. GEFs promote the exchange of GTP for GDP and transition to the activated state. 2) A receptor-derived signal may alter the activity of a GTPase activating protein (GAP) which enhances the intrinsic hydrolytic activity of the GTPase. The data presented in this chapter examines FcεR1 regulation of an adapter molecule/GEF complex and presents a model for the membrane proximal signalling cassette which couples the FcεR1 to Ras family GTPases.

3.1.1 Adapter molecule coupling to GTPase/kinase cascades; paradigms from *Drosophila* and mammalian growth factor receptors.

Detailed genetic analyses have been performed on signalling pathways which couple receptors to GTPases in several systems including *Drosophila, C. elegans* and mammalian cells. These data have revealed a common signalling cassette used to couple transmembrane receptors to GEFs and hence GTPases. Briefly, ligation of a receptor leads to activation of either intrinsic or associated tyrosine kinase activity. One or more adapter molecules then transduce the activatory signal between the tyrosine kinase activity and effector proteins. Typically, an SH2 domain containing adapter protein binds phosphorylated tyrosine residues in the aggregated receptor itself or in another adapter protein which is a substrate for receptor associated tyrosine kinases. The adapter brings effector molecules to the membrane. When the effector molecule is

a GEF, then the net result of this process is the activation of a GTPase and hence initiation of a cascade of serine/threonine kinase activities leading eventually to the activation of a mitogen activated protein kinase (MAPK) family members.

This mechanism for coupling of transmembrane receptors to GEFs and GTPases is highly conserved. Figure 3.1 shows the components of such cassettes in *Drosophila, C. elegans* and the vertebrate epidermal growth factor receptor. These signaling cassettes are reviewed in (Dickson and Hafen, 1994; Hafen *et al.*, 1993).

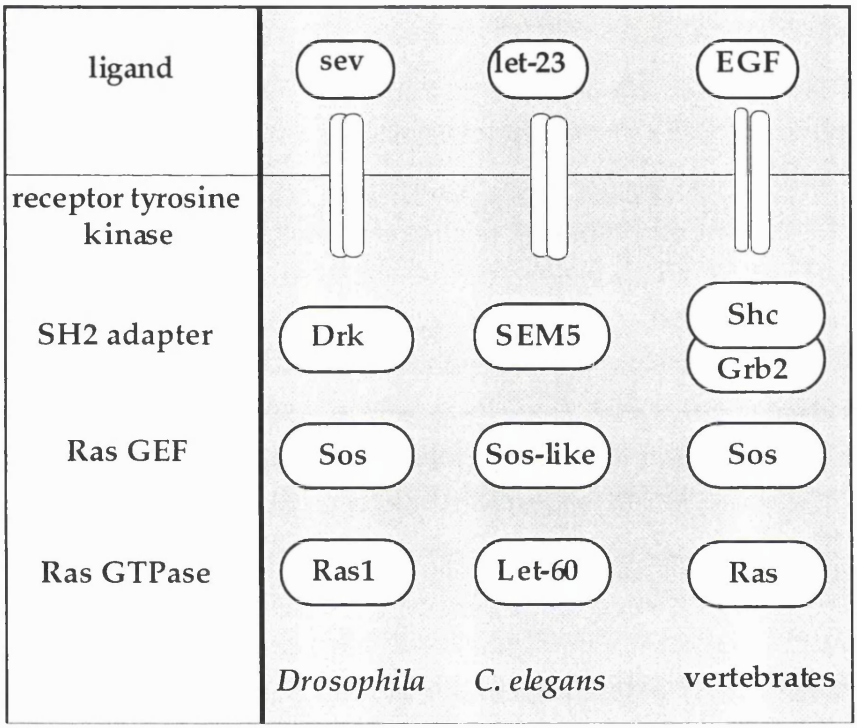


Figure 3.1. Adapter cassettes couple transmembrane receptors to Ras family GTPases. Ligand dependent aggregation of receptor tyrosine kinases results in recruitment of SH2 domians in the adapter molecule to phosphoacceptor sites in the receptor cytoplasmic tail. Adapter molecules are complexed with Ras GEFs via their effector domains, membrane recruitment allows GEF interaction with GTPase substrate and hence GTP loading of the GTPase. GEF: guanine nucleotide exchange factor, EGF: epidermal growth factor. From (Hafen *et al.*, 1993).

Regulation of R7 photoreceptor cell fate in *Drosophila* eye is regulated by the sevenless (sev) receptor tyrosine kinase. This pathway regulates Ras via the SH2/SH3 domain containing Drk adapter and the Sos GEF. In *C. elegans* a similar cassette targets Ras and is used to determine vulval cell fate. There are a number of counterparts for these cassettes in higher organisms. In mammalian systems such as growth factor receptor and antigen receptor signalling, these cassettes centre upon protein complexes formed around the Grb2 adapter. There is considerable diversity in

the protein-protein interactions which can link a receptor to Grb2 and its effector proteins. Those identified to date fall into distinct categories:

1) *Direct recruitment to a receptor tyrosine kinase.* Ligation and aggregation of receptors with intrinsic tyrosine kinase activity results in phosphorylation of consensus binding sites for the Grb2 SH2 domain and hence Grb2 recruitment. The mammalian Epidermal Growth Factor Receptor (EGFR) recruits Grb2 to autophosphorylation sites in its cytoplasmic tail and hence is linked to Sos and Ras . The Tyr at position 1068 is able to directly recruit Grb2 from the cytoplasm of Cos-7 cells. These data suggest that recruitment of Grb2 directly to activated EGFR aggregates is a major mechansim by which EGF couples to the Ras pathway (Buday and Downward, 1993; Chardin *et al.*, 1993; Egan *et al.*, 1993).

2) *Recruitment via an intermediary adapter.* This mechanism is seen in both growth factor receptor systems and where receptors have no intrinsic tyrosine kinase activity. In the EGFR system, the Shc adapter protein (described below) links via its PTB domain to phosphorylated tyrosine residues in aggregated receptors (Egan *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Skolnik *et al.*, 1993). Shc is in a complex with Grb2 and hence recruits Grb2 and associated effector proteins. This interaction is apparently in addition to the direct recruitment of Grb2 to the EGFR observed in certain cells. The TRK receptor for Nerve Growth Factor (NGF) contains a high affinity binding site for Shc at position Y490, which is part of a consensus NPXpY motif (Mandiyan *et al.*, 1996; van der Geer *et al.*, 1995). Mitogenic signalling by TRK occurs without a detectable TRK/Grb2 interaction. Rather, Ras dependent MAP kinase activation involves Shc acting as an adapter betweeen TRK and Grb2 (Nakamura *et al.*, 1996).

In B and T cells, antigen receptor aggregation results in activation of receptor associated tyrosine kinases. Phosphorylated ITAM motifs provide docking sites for adapter molecules which may be substrates for receptor associated tyrosine kinases. In the T cell antigen receptor sytstem, a putative adapter molecule, p36, is tyrosine phosphorylated in response to TCR ligation. The p36 protein associates with the Grb2 SH2 domain, recruiting Grb2 and its SH3 bound proteins to the plasma membrane. In the FcεR1 system there has been no analysis of Grb2 interactions.

3.1.2 The adapter molecule Grb2 is composed exclusively of SH2 and SH3 domains.

Grb2 (Growth-factor Receptor Binding protein-2) is an adapter protein composed of one SH2 and two SH3 domains. The gene for Grb2 was cloned initially in *C. elegans* (Clark *et al.*, 1992). The human and mouse homologues display approximately 60% sequence homology with this gene, but more importantly have the

same spatial distribution of signalling domains. This modular design permits interaction with phosphorylated tyrosine residues via the SH2 domain, on proteins which have been substrates for receptor-activated protein tyrosine kinases (PTKs).

Mutagenesis studies using phosphopeptides spanning putative SH2 binding sites have revealed that individual SH2 domains have defined sequence requirements in their binding partners (Songyang *et al.*, 1993; Songyang *et al.*, 1994). They have no significant affinity for unphosphorylated Tyr residues. The binding affinity of SH2 for phosphopeptides has K_d=10-100nM for optimally designed peptides. Residues which flank the target pTyr can increase this affinity to the pM level. Hence, in binding of a target molecule to an SH2 domain there is a binary input of the phosphorylation status of the target Tyr, and subsequent tuning of affinity derived from the residues flanking the Tyr. The SH3 domains have affinity for proline-rich regions in effector molecules. SH3 binding sites are composed of proline rich decapeptides. These peptides bind to SH3 domains with K_d=5-100μM. Specificity is apparently conferred by interaction of non-proline residues in the ligand and variable loop regions in the SH3 domain. By virtue of its modular composition, Grb2 is therefore in a position to nucleate the formation of protein complexes.

3.1.3 Shc is composed of multiple protein signalling domains.

A variety of Shc isoforms exist in mammalian cells. Shc proteins are adapter molecules with no intrinsic enzymatic activity(Pelicci *et al.*, 1992). They are composed of various protein signalling modules, including a PTB (phosphotyrosine binding) domain, one or more proline rich regions, which may dock SH3 domains, and an SH2 domain. Shc is known to be tyrosine phosphorylated in response to cytokine receptor ligation (e.g. IL-2R in T cells, IL-3R and GM-CSFR in mast cells). Shc is strongly tyrosine phosphorylated in response to BCR stimulation of B cells (Saxton *et al.*, 1994; Smit *et al.*, 1994) but only weakly tyrosine phosphorylated following TCR ligation (Osman*et al.*, 1995; Sieh *et al.*, 1994). The status of Shc in FcεR1 stimulated mast cells has not been addressed. Shc has been primarily described as an adapter linking mammalian growth factor receptors to the regulation of Grb2/Sos complexes. As described below, it may also be important in this regard in antigen receptor regulated leukocytes.

3.1.4 Grb2 complexes in antigen receptor mediated signal transduction.

Membrane proximal signalling by the B and T cell antigen receptors has been extensively studied. Antigenic ligation of these receptors results in the expression of common functions with mast cells; there are secretory responses (e.g. from $CD8^+$ cytotoxic T cells) and transcriptional induction of multiple gene targets. A number of published studies have shown that Grb2 protein complex formation is a common early

signalling mechanism used by leukocyte antigen receptors. The data from these studies have identified a number of components of Grb2 complexes and are summarised below. There is extensive literature on potential Grb2 interactions identifed using yeast two hybrid or fusion protein affinity matrix systems. A subset of these interacting proteins have been shown to associate with endogenous Grb2 and are thus known to be relevant *in vivo*. Only interactions which have been shown to occur with endogenous cellular Grb2 are listed.

1) *Grb2 SH2 associated proteins.* In B cells the Shc adapter is found in complex with Grb2 SH2 domains. Shc apparently binds directly to phosphorylated ITAMs in the BCR complex. Shc tyrosine phosphorylation is BCR induced and hence Shc seems to be the major linkage between the BCR and Ras (Gold *et al.*, 1993; Saxton *et al.*, 1994; Smit *et al.*, 1994). In T cells there is a more diverse range of proteins associated with the Grb2 SH2 domain. Shc/Grb2 complexes are found but the major adapter between TCR ITAMs and Grb2 is an uncharacterised 36kDa tyrosine phosphoprotein, p36 (Buday *et al.*, 1994). The identity of this protein is a contentious issue. A 36kDa protein, lnk, was recently cloned from rat thyroid library (Huang *et al.*, 1995; Takaki *et al.*, 1997) and can associate with Grb2 SH2 domain fusion proteins. However, anti-lnk antibodies do not recognise the p36 found in complex with endogenous Grb2 in T cells. Finally, the Syp phosphatase can be isolated with Grb2 SH2 domains in Jurkat T cells (Marengere *et al.*, 1996). Whilst this implies that Syp can recruit Grb2/Sos, it is not clear which downstream effectors are selected for by a Syp/Grb2 complex and hence which biochemical pathways are initiated in a Syp dependent manner.

2) *Grb2 SH3 associated proteins.* In all systems examined to date the Sos GEF is found complexed to Grb2 SH3 domains. This association is not dependent on receptor activation. Grb2 SH3 domains in the B cells also complexes with two inducibly tyrosine phosphorylated species of 130 and 145kDa (Saxton *et al.*, 1994; Smit *et al.*, 1994). It has been suggested that the 130kDa protein may be the product of the *cbl* oncogene. Reif *et al* show that there is another postulated effector molecule for Grb2 in T cells (Reif *et al.*, 1994). These data show that a 75kDa tyrosine phosphoprotein, later cloned and named SLP-76 (SH2 containing leukocyte protein of 76 kDa) molecule, binds the Grb2 SH3 domain (Jackman *et al.*, 1995; Motto *et al.*, 1996). SLP-76 is a haematopoietic cell specific protein containing numerous phosphoacceptor sites in the N-terminus, a central region rich in proline residues (a potential docking site for SH3 domains) and a C-terminal SH2 domain.

Clearly, Grb2 is a versatile adapter used by antigen receptors to connect receptor-associated PTKs to multiple signalling pathways. The FcεR1 is a member of

the leukocyte antigen receptor superfamily. In the context of the above literature on the BCR and TCR, it was not known if common signalling elements were shared by the FcεR1. It seemed reasonable to postulate that mast cells would use a Grb2 cassette to accomplish regulation of Ras via Sos. Grb2 might also link the FcεR1 to non-Ras pathways. The first step in understanding the significance of any such linkage lay in identifying the components of Grb2 complexes in the FcεR1 system. This type of study would help to move the knowledge of this system forward towards a comparable state with understanding of TCR and BCR signalling. Therefore the following questions were asked:

1) Is the Grb2/Sos complex a target for FcεR1 signals in RBL2H3?

2) What is the mechanism of linkage between the FcεR1 and Grb2?

3) What effector molecules bind Grb2 in RBL2H3?

3.2 RESULTS

3.3. The major components of Grb2 signalling complexes are present in RBL2H3 cells.

The objective of the work presented in this chapter was to analyse the components of protein complexes formed around the adapter molecule Grb2 in RBL2H3 cells. Initial experiments therefore used Western blotting to assess if any of the components of Grb2 complexes characterised in other leukocytes were present in RBL2H3.

The data in Figure 3.2 show Western blot analyses of post-nuclear cell lysate from RBL2H3 and, as a comparison, the Jurkat leukaemic T cell line. Grb2 itself and its putative binding partners Shc and Sos are present in RBL2H3 and Jurkat, as is the Sos target, p21 Ras. At the time these experiments were performed, the SLP-76 molecule had not been cloned and no anti-sera were available. Later experiments confirmed SLP-76 expression in RBL2H3 (Figure 3.16, (Hendricks-Taylor *et al.*, 1997)). These data on expression confirmed that it was reasonable to embark upon a study of FcεR1 regulation of Grb2 complexes in RBL2H3.

3.4 Fusion proteins and affinity matrices purify Grb2 SH3 domain associated Sos from RBL2H3.

As described in the Introduction, the Grb2 adapter protein couples a variety of receptors to the Sos GEF molecule. The experiments shown in this section asked if the Grb2/Sos complex existed in RBL2H3 and hence if the FcεR1 was likely to regulate Ras via this mechanism in common with other antigen receptors. To assess whether Sos and Grb2 interact in RBL2H3 two approaches were used. 1) A GST fusion protein of the full-length Grb2 molecule was used as an affinity matrix and 2) two reciprocal reagents were used as affinity matrices to assess the SH domain specificity of any Grb2/Sos interaction. These reagents are described below and schematically in Figure 3.3.

a) *GST fusion protein of wild-type Grb2.* When used as an affinity matrix this fusion isolates proteins which are capable of interacting with any part of the Grb2 molecule.

b) *EGFR-Y1068 phosphopeptide.* A synthetic tyrosine phosphopeptide corresponding to the autophosphorylation site Tyr-1068 in the epidermal growth factor receptor (EGFR-Y1068) was used to precipitate Grb2 from RBL2H3 cells. The EGFR-Y1068 peptide binds to the Grb2 SH2 domain with nanomolar affinity and thus competitively blocks association between cellular proteins and the SH2 domain of Grb2. Use of this peptide as an affinity matrix purifies Grb2 and proteins bound only

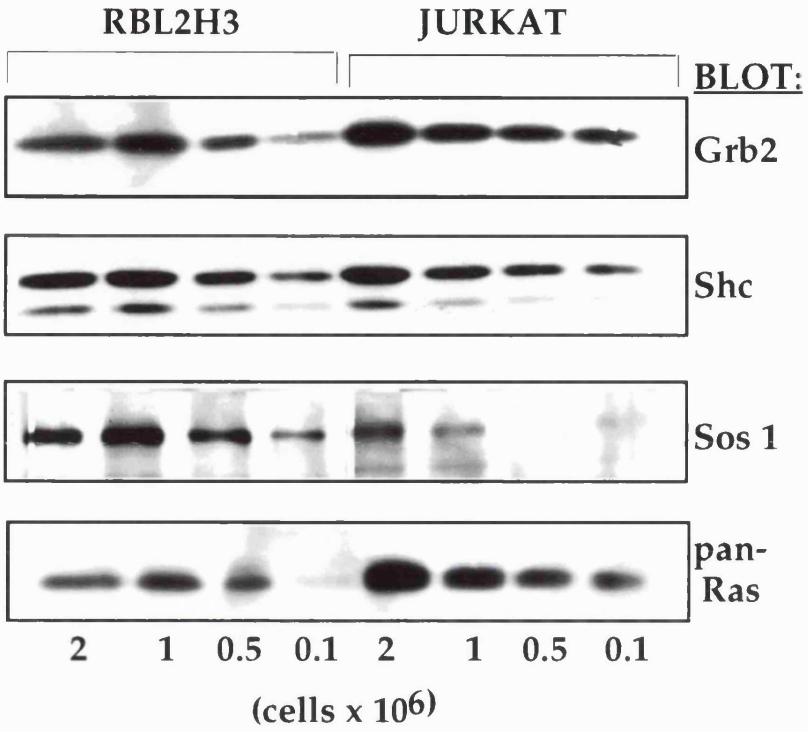


Figure 3.2. Components of Grb2 protein complexes are present in RBL2H3. Post-nuclear lysate from RBL2H3 and Jurkat T cells was prepared as described in Materials and Methods. After acetone precipitation proteins were resolved by 7-17% gradient SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in 5% non-fat milk before probing with monoclonal anti-Grb2 (0.5µg/ml), polyclonal anti-Shc (1µg/ml), polyclonal anti-mSos1 (2µg/ml) or anti-pan Ras (0.1µg/ml). Numbers of cell equivalents loaded per lane are indicated.

to the Grb2 SH3 domains and competitively prevents isolation of proteins binding the Grb2 SH2 domain.

c) *GST-Sos fusion protein.* GST-Sos comprises a fusion of a proline rich fragment (amino acids 1135-1336) from the carboxyl-terminal region of Sos-1. Sos-1 is a known Grb2 effector molecule in various cell types. The GST-Sos fusion protein isolates Grb2 and proteins associated with the Grb2 SH2 domain, whilst competitively preventing the copurification of SH3 binding proteins.

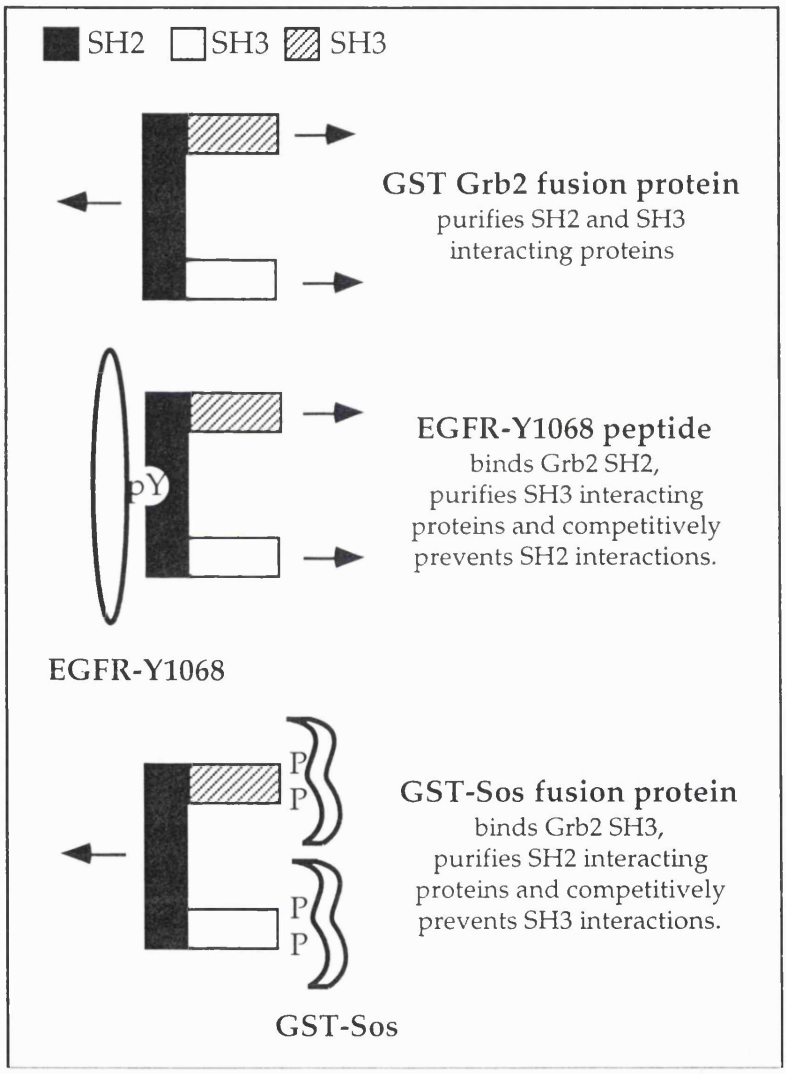


Figure 3.3. Reagents used as affinity matrices to purifiy Grb2 interacting proteins from RBL2H3.

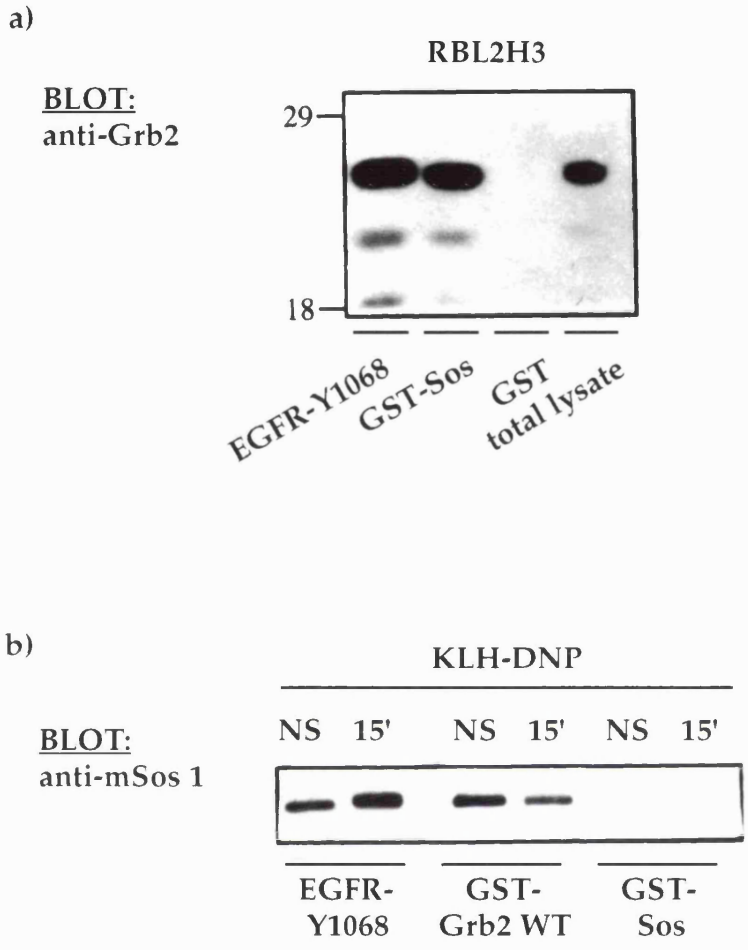


Figure 3.4. a) The EGFR-Y1068 phosphopeptide and GST-Sos fusion protein purify the majority of endogenous Grb2 from RBL2H3 when used as affinity matrices. Affinity purifications from $1x10^7$ RBL2H3 per lane and acetone precipitation of post-nuclear protein from $1x10^6$ RBL2H3 were carried out as described in Materials and Methods. Proteins were resolved by 15% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in 5% non-fat milk before probing with monoclonal anti-Grb2 (0.5µg/ml). Molecular weights are indicated in kDa. **b) Sos is complexed to the SH3 domains of endogenous Grb2 in RBL2H3.** $1x10^7$ RBL2H3 per lane were left unstimulated (NS) or primed with 1µg/ml IgE anti-DNP (1h, 37°C) before stimulation for 15 min at 37°C with 500ng/ml KLH-DNP. Cells were lysed and affinity purifications were carried out as described in Materials and Methods. Proteins were resolved by 10% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in 5% non-fat milk before probing with polyclonal anti-mSos 1 (2µg/ml).

The efficacy of the EGFR-Y1068 peptide and GST-Sos fusion protein as affinity matrices for Grb2 purification was assessed. The data in Figure 3.4a show that EGFR-Y1068 and GST-Sos purify the majority of endogenous Grb2 from cellular lysates. This figure is a Western analysis of EGFR-Y1068 and GST-Sos affinity purifications performed with a monoclonal Grb2 antibody. Both reagents clearly purify the majority of endogenous Grb2 from lysate of $1x10^7$ cell equivalents, as shown by comparison with the amount of Grb2 in total lysate from $1x10^6$ cell equivalents.

These reagents and the GST-Grb2 fusion protein were then used to assess whether Grb2/Sos complexes were present in RBL2H3. An anti-Sos Western blot was performed to assess whether Sos binds to the Grb2 SH3 domains. The data in Figure 3.4b show that Sos is present in EGFR-Y1068 and GST-Grb2 affinity purifications prepared from RBL2H3. Moreover, Sos can be co-purified with the endogenous Grb2 protein isolated with the EGFR-Y1068 phosphopeptide. Sos does not co-purify with the Grb2 complexes isolated using the GST-Sos fusion protein. Therefore, Sos is associated with the SH3 domains of Grb2 in mast cells but cannot bind to the Grb2 SH2 domain. There is a basal association of Sos with the Grb2 SH3 domain which is apparently enhanced in FcεR1 stimulated RBL2H3.

Sos is in a complex with the Grb2 SH3 domains in RBL2H3. A panel of fusion proteins analogous to the full-length Grb2 was used to assess whether Sos exhibited any SH3 domain preference. The nature of these GST fusions is shown in Figure 3.5. Affinity binding experiments were performed with truncated or mutated versions of the Grb2 fusion protein comprising the isolated amino- and carboxyl-terminal SH3 domain, called GST-Grb2NSH3 (amino acids 1-58) and GST-Grb2CSH3 (amino acids 159-217) respectively. In addition, GST-Grb2 with mutations introduced into both SH3 domains was used (GST-Grb2μSH3). The amino acid substitutions (P49L and G203R) in the SH3 domains of GST-Grb2μSH3 ablate their binding capability but leave the ability to form SH2 mediated interactions intact.

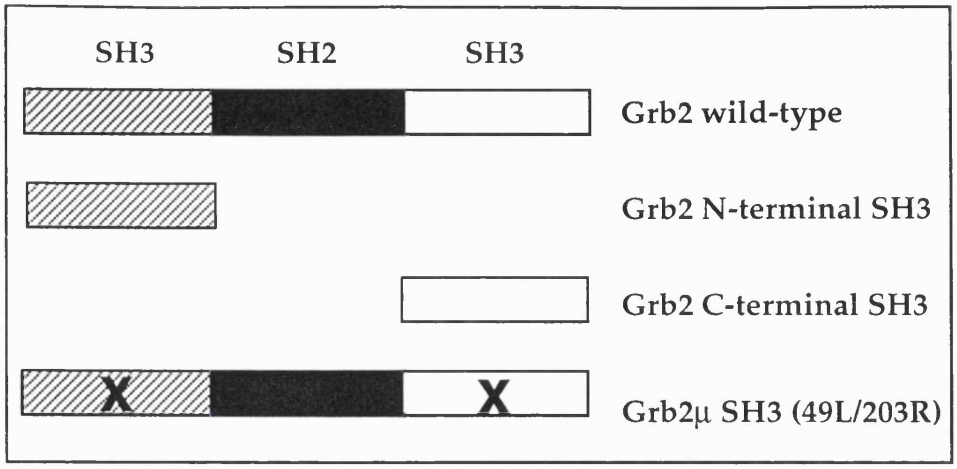


Figure 3.5. GST fusion proteins of Grb2. Full length Grb2 will purify both SH2 and SH3 interacting proteins from cell lysates. The isolated N- and C-terminal SH3 domains can be used to assess any preferential binding. The Grb2μSH3 protein contains point mutations in both SH3 domains which ablate binding. Hence Grb2μSH3 will purify only SH2 dependent interactions.

The data in Figure 3.6 show an anti-Sos Western analysis of GST-Grb2 fusion protein affinity purifications prepared from RBL2H3 cells. Sos is found binding to the full length Grb2 and N-terminal SH3 domain fusions in the absence and presence of FcεR1 stimulation of RBL2H3. Sos shows a marked preference for the N-terminal SH3 domain of Grb2; low levels of Sos are found in complex with the C-terminal SH3 domain fusion.

The data presented above suggest that in common with other cell systems, RBL2H3 contain Grb2/Sos complexes. Such complexes could provide the mechanism by which the FcεR1 regulates the guanine nucleotide binding status of Ras, in a manner analogous to that employed by the B and T-cell antigen receptors. Two important questions arose from these experiments; 1) Is the Grb2/Sos complex regulated by the FcεR1? and 2) What is the mechanism by which the FcεR1 couples to Grb2/Sos?

3.5 Antigenic crosslinking of the FcεR1 induces multiple protein tyrosine phosphorylations.

The possibility that tyrosine phosphoproteins are important components of the Grb2 complexes in RBL2H3 was explored. The data Figure 3.7 show a Western blot analysis with anti-phosphotyrosine antibodies of total cellular lysate from quiescent and FcεR1 stimulated RBL2H3 cells. There is very little basal tyrosine phosphorylation of proteins in the absence of stimulation. However, crosslinking of the FcεR1 using multivalent antigen results in prominent tyrosine phosphorylations of proteins at the

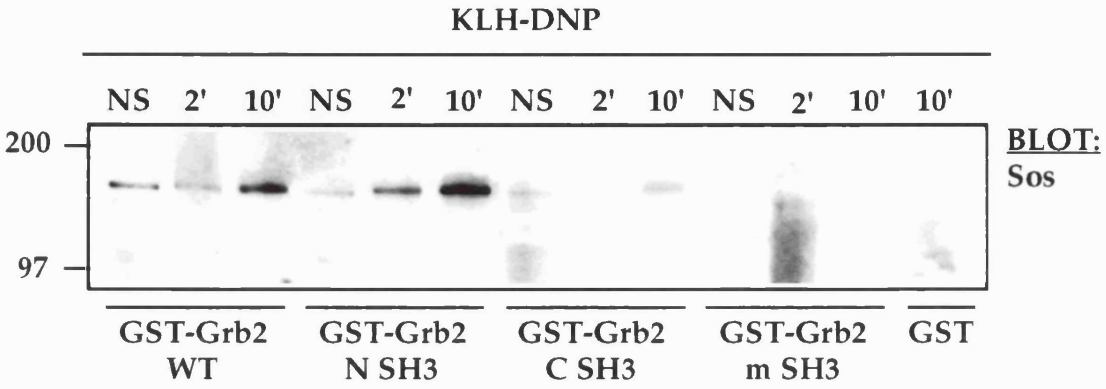


Figure 3.6. Sos exhibits markedly preferential binding to the amino-terminal SH3 domain of Grb2. $1x10^7$ RBL2H3 per lane were left unstimulated (NS) or primed with 1µg/ml IgE anti-DNP (1h, 37°C) before stimulation for 2 min or 10 min at 37°C with 500ng/ml KLH-DNP. Affinity purifications were carried out as described in Materials and Methods using fusion proteins of full length Grb2 (GST-Grb2 WT), isolated amino- (GST-Grb2 NSH3) or carboxy-terminal (GST-Grb2 CSH3) Grb2 SH3 domains or the double SH3 mutant (GST-Grb2 mSH3) which purifies SH2 binding proteins. Proteins were resolved by 11% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in 5% non-fat milk before probing with polyclonal anti-mSos 1 (2µg/ml). Molecular weights are indicated in kDa.

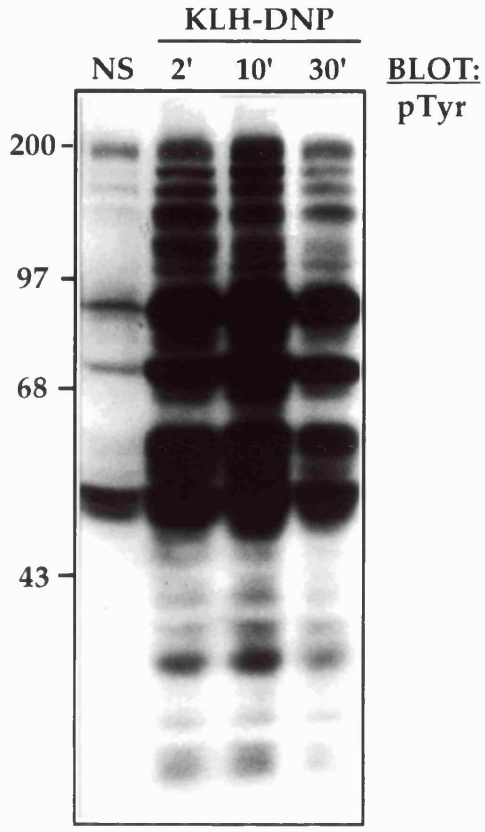


Figure 3.7. Antigenic crosslinking of the FcεR1 in RBL2H3 induces multiple protein tyrosine phosphorylations. $1x10^6$ RBL2H3 per lane were left unstimulated (NS) or primed with 1μg/ml IgE anti-DNP (1h, 37°C) before stimulation for 2 min, 10 min or 30 min at 37°C with 500ng/ml KLH-DNP. Cells were lysed and post-nuclear proteins were acetone precipitated as described in Materials and Methods. Proteins were resolved by 11% SDS-PAGE and transferred to PVDF membrane. Membrane was blocked in 5% non-fat milk before probing with monoclonal anti-phosphotyrosine antibody (4G10) at 1μg/ml. Molecular weights are indicated in kDa.

following molecular weights; 70-75kDa, 60-65kDa and 50-55kDa. Tyrosine phosphorylations were rapidly induced, detected within 2 min and sustained for a period of 30 min. In control experiments, no increase in tyrosine phosphorylation was observed over this time period in lysate from cells which had been incubated with monomeric IgE but not exposed to crosslinking multivalent antigen (data not shown).

These data show that induction of tyrosine kinase activity is a major consequence of antigenic crosslinking of the FcεR1. A number of these tyrosine phosphorylated species had previously been identified by other groups. Published data showed that the 72kDa Syk kinase is heavily tyrosine phosphorylated after FcεR1 crosslinking . In the 50-55kDa region of the gel shown it is likely that one prominent species is the FcεR1 associated *src* kinase lyn. Phospholipase Cγ1 (145kDa), Vav (95kDa), ERK kinases (42-44kDa) and the FcεR1β subunit (30kDa) have also been identified previously as substrates for FcεR1 associated PTKs (Benhamou *et al.*, 1993; Benhamou *et al.*, 1992; Jouvin *et al.*, 1994; Park *et al.*, 1991a; Rivera *et al.*, 1988; Turner *et al.*, 1995a). However, many tyrosine phosphorylations remain unassigned to individual species and these data do not approach the question of whether any protein substrates for FcεR1 associated PTKs are complexed with Grb2 in RBL2H3.

3.6 Multiple tyrosine phosphoproteins associate with GST-Grb2 fusion proteins in RBL2H3.

Grb2 fusion proteins were used as affinity matrices in initial experiments to examine whether proteins complexed with Grb2 are regulated by FcεR1 associated tyrosine kinases. The affinity matrices used for binding experiments comprised Glutathione S-transferase (GST) fusions of full length or truncated Grb2 molecules, coupled to Glutathione agarose beads. The data in Figure 3.8 show an anti-phosphotyrosine Western analysis of proteins binding to the wild-type GST-Grb2 fusion protein in RBL2H3 mast cells. There are multiple tyrosine phosphoproteins in protein complexes purified with GST-Grb2 from both quiescent and FcεR1-activated RBL2H3 cell lysates. Many proteins are observed which are basally tyrosine phosphorylated in unstimulated cells. However, there is a significant FcεR1 dependent increase in tyrosine phosphorylation of proteins migrating at 120,110, 75,65 and 30-33kDa. The experiment in Figure 3.8 also explores the SH domain specificity of FcεR1 induced Grb2 binding tyrosine phosphoproteins.

The data show that a prominent FcεR1-induced tyrosine phosphoprotein of 75kDa was purified by wild type Grb2 and isolated Grb2 SH3 domains. Both GST-Grb2 SH3 domain fusion proteins also purified tyrosine phosphoproteins of 38, 52-55, 120 and 140kDa. Neither the 75kDa or the latter species bound to the GST-Grb2μSH3 fusion protein. Antigenic crosslinking of the FcεR1 induced tyrosine

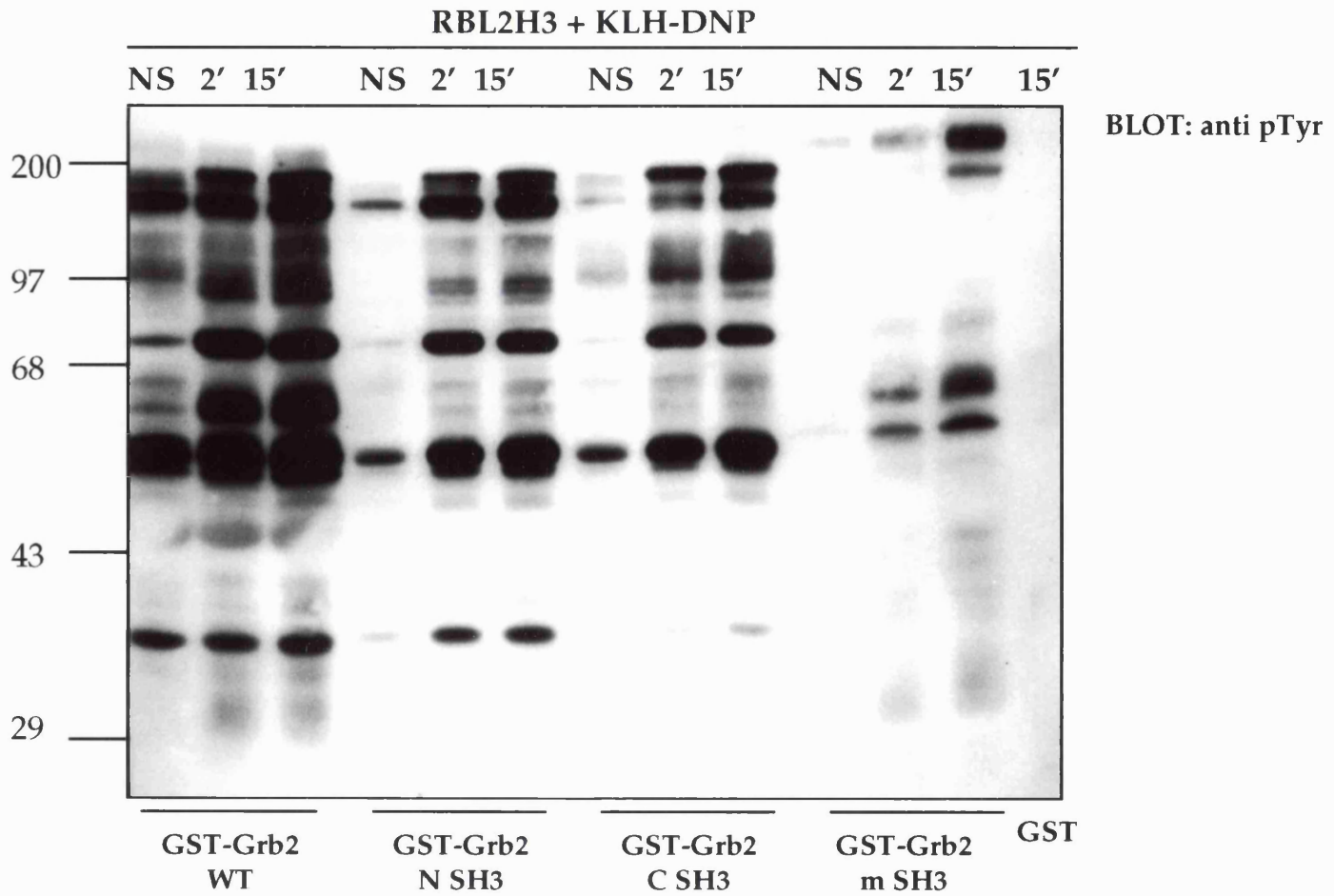


Figure 3.8. SH domain specificity of tyrosine phosphoproteins binding to Grb2 fusion proteins in RBL2H3. RBL2H3 were primed as described and stimulated with 500ng/ml KLH-DNP for the indicated times. Lysates were affinity purified for 2h with the indicated GST-Grb2 fusion protein. GST alone was included as a negative control. Samples were resolved under reducing conditions by 11% SDS-PAGE before transfer to PVDF membrane and anti-phosphotyrosine Western analysis using 1µg/ml 4G10.

phosphorylation of 33 and 65kDa proteins which bound to the wild-type GST-Grb2 fusion protein. These latter proteins did not bind to the isolated Grb2 SH3 domains but bound efficiently to the GST-Grb2μSH3 fusion protein. In addition, a 55kDa tyrosine phosphoprotein and a doublet of high molecular weight (120-140kDa) tyrosine phosphoproteins were detected binding to GST-Grb2μSH3.

A 35kDa inducibly tyrosine phosphorylated species exhibited a marked preference, for the N-terminal SH3 domain of Grb2 but other protein did not appear to be strongly selective for a particular SH3. From these binding experiments it can be concluded that there are multiple substrates for FcεR1 activated tyrosine kinases which bind to Grb2 fusion proteins. FcεR1 induced tyrosine phosphoproteins of 33 and 65kDa bind to Grb2 SH2 domains. There is a major 75kDa species binding to the Grb2 SH3 domains. In addition, there are multiple tyrosine phosphoproteins of 110-120kDa which associate with the Grb2 SH3 domains and of 52-55 and 120-140kDa which can bind to the SH2 domain. From the binding experiments shown here, it is clear that the latter species can be resolved into those which are basally phosphorylated and substrates for FcεR1 activated tyrosine kinases.

3.7 Several tyrosine phosphoproteins associate with the Grb2 SH2 domains in RBL2H3.

Analysis of Grb2 complexes was next directed towards the SH2 binding partners of Grb2. The GST-Grb2μSH3 fusion protein has disruptive mutations in both SH3 domains, therefore any interactions can only reflect binding via the Grb2 SH2 domain. The data in Figure 3.8 have shown that use of the GST-Grb2μSH3 fusion protein as an affinity matrix selects for a small number of the phosphoproteins shown to bind the full-length GST-Grb2. GST-Grb2μSH3 binds prominent species of 120-140kDa and 55kDa which are constitutively tyrosine phosphorylated. In addition, there are proteins of 60-62kDa and 33kDa which are tyrosine phosphorylated in response to antigenic crosslinking of the FcεR1.

As described above, the GST-Sos fusion protein affinity purifies endogenous Grb2 via its SH3 domains, co-purifying proteins associated with the Grb2 SH2 domain. The data in Figure 3.9(a) show that GST-Sos purifies Grb2 with two associated tyrosine phosphoproteins from RBL2H3. These are a 55kDa constitutively tyrosine phosphorylated protein and a 33kDa molecule which is a substrate for FcεR1 activated tyrosine kinases. Further experiments were directed towards the identification of these proteins.

3.8 Shc is complexed to the Grb2 SH2 domain in mast cells but is not a substrate for FcεR1 regulated PTKs.

In many cell types the adapter molecule Shc is tyrosine phosphorylated in response to receptor stimulation and forms a complex with Grb2 via the SH2 domain. Shc-Grb2-Sos complexes are thought to be a critical regulatory link between receptor tyrosine kinases and activation of the Ras GTPase. Shc proteins occur in 46, 55 and 62kDa isoforms. Therefore, Shc was a candidate for the 55kDa tyrosine phosphoprotein found in complex with the Grb2 SH2 domain in RBL2H3 cells.

Figure 3.9(a) shows anti-phosphotyrosine Western analysis of affinity purifications carried out using GST-Grb2 and GST-Grb2μSH3. The GST-Sos fusion protein was also used to purify endogenous Grb2 and associated tyrosine phosphoproteins. Figure 3.9(b) shows a Western analysis of the same membrane using a polyclonal antisera to Shc. The data show that Shc binds the Grb2 SH2 domain in vitro and in vivo, since it is found complexed to Grb2 purified using the GST-Sos fusion protein. This indicates that Shc is a binding partner of the endogenous Grb2 SH2 domain. However, these preliminary data suggested that Shc tyrosine phosphorylation was not altered by the activation status of the RBL2H3, a situation not reflected in the T and B-cell antigen receptor systems. Therefore, the tyrosine phosphorylation of Shc was analysed further.

The Trk-Y490 tyrosine phosphopeptide was used as an affinity matrix for Shc purification. This peptide corresponds to a high affinity binding site for Shc PTB domains derived from the Nerve Growth Factor receptor. The data in Figure 3.10 show that Trk-Y490 is a highly effective reagent for purification of Shc from cell lysates, purifying the major part of both 55 and 46kDa Shc isoforms from $1x10^7$ RBL2H3 cells. The total Shc protein present in $1x10^6$ RBL2H3 cell equivalents is shown as a comparison.

Figure 3.11 shows affinity purifications from RBL2H3 cells using Trk-Y490, GST-Grb2 and the GST-Sos fusion protein. Western analysis was carried out with anti-phosphotyrosine antibody. The 55kDa tyrosine phosphoprotein binding to GST-Grb2 and GST-Sos comigrates with the 55kDa Shc isoform purified by Trk-Y490. Moreover, the 55kDa Shc isoform is tyrosine phosphorylated in both quiescent and FcεR1 stimulated RBL2H3 cells. Taken together these data indicate that, in common with other systems, Shc is found complexed to the Grb2 SH2 domain in RBL2H3 cells. However, Shc is not a substrate in this system for receptor activated protein tyrosine kinases.

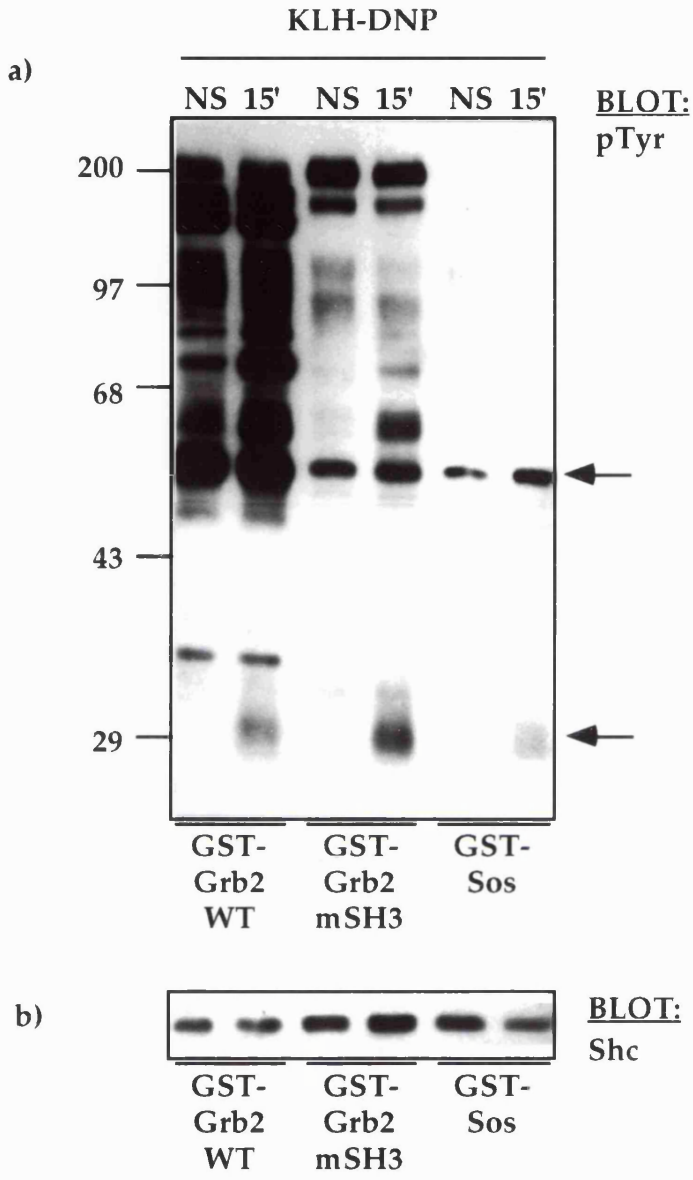


Figure 3.9. a) GST-Sos purifies Grb2 with two SH2 associated tyrosine phosphoproteins of 55 and 33kDa. $1x10^7$ RBL2H3 per lane were left unstimulated (NS) or primed with 1µg/ml IgE anti-DNP (1h, 37°C) before stimulation for 15 min at 37°C with 500ng/ml KLH-DNP. Affinity purifications were carried out as described in Materials and Methods using fusion proteins of full length Grb2 (GST-Grb2 WT), double SH3 mutant (GST-Grb2 mSH3) or GST-Sos which purifies SH2 binding proteins. Proteins were resolved by 11% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in 5% non-fat milk before probing with monoclonal anti-phosphotyrosine (4G10) (1µg/ml). Arrows mark the positions of the 55kDa and 33kDa tyrosine phosphoproteins in complex with the Grb2 SH2 domain. **b) Shc is constitutively associated with the Grb2 SH2 domain in RBL2H3.** The membrane from (a) was reprobed with polyclonal anti-Shc (1µg/ml). Molecular weights are indicated in kDa.

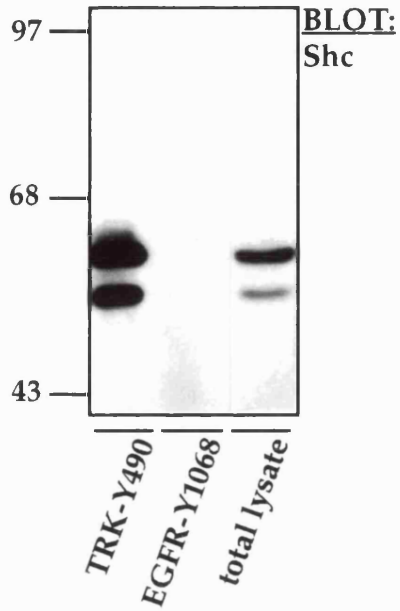


Figure 3.10. The TRK-Y490 phosphopeptide purifies Shc when used as an affinity matrix. Affinity purifications were carried out from $1x10^7$ RBL2H3 per lane as described in Materials and Methods using affinity matrices of TRK-Y490 or EGFR-Y1068 phosphopeptides. Post-nuclear protein was acetone precipitated from lysate of $1x10^6$ RBL2H3 (total lysate). Proteins were resolved by 11% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in 5% non-fat milk before probing with polyclonal anti-Shc (1µg/ml). Molecular weights are indicated in kDa.

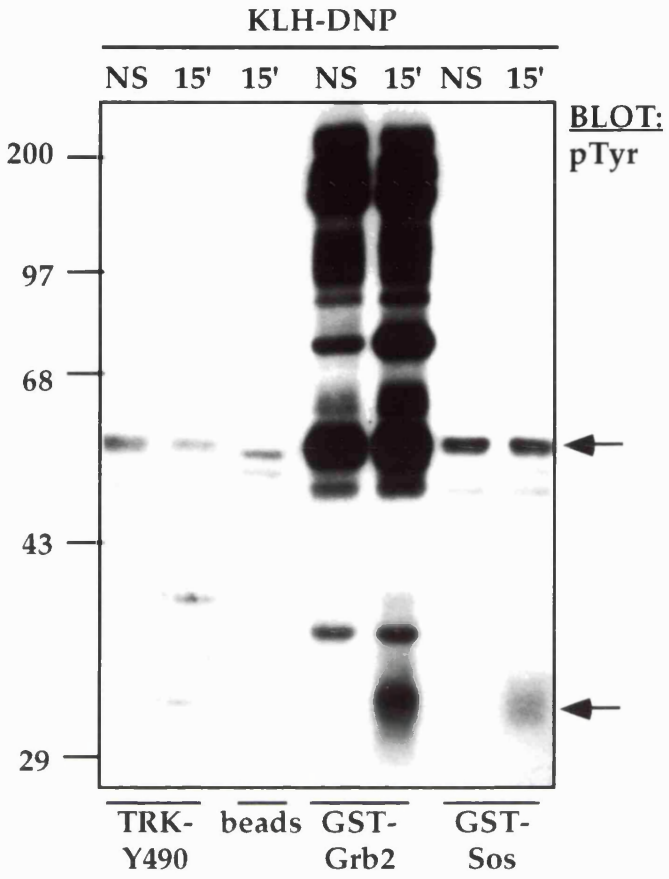


Figure 3.11. The 55kDa tyrosine phosphoprotein associated with the Grb2 SH2 domain in RBL2H3 is Shc. $1x10^7$ RBL2H3 per lane were left unstimulated (NS) or primed with 1µg/ml IgE anti-DNP (1h, 37°C) before stimulation for 15 min at 37°C with 500ng/ml KLH-DNP. Affinity purifications were carried out as described in Materials and Methods using affinity matrices of the TRK-Y490 phosphopeptide coupled to Affigel-10 beads, Affigel-10 beads alone (beads) or fusion proteins of full length Grb2 (GST-Grb2) and GST-Sos. Proteins were resolved by 11% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in 5% non-fat milk before probing with monoclonal anti-phosphotyrosine (4G10) (1µg/ml). Arrows mark the positions of Shc and the 33kDa tyrosine phosphoprotein in complex with the Grb2 SH2 domain. Molecular weights are indicated in kDa.

3.9 p33 is neither the T cell p36 molecule or the FcεR1 β sub-unit.

The accumulated evidence presented so far shows that FcεR1 triggering results in the tyrosine phosphorylation of p33, which binds to the Grb2 SH2 domain. The identity of this p33 molecule is not known. The β chain of the FcεR1 is a 30kDa molecule which is tyrosine phosphorylated in response to FcεR1 crosslinking, primarily in the ITAM domain of its C-terminal cytoplasmic tail. Accordingly, it was possible that p33 was in fact the tyrosine phosphorylated FcεR1β. This question was addressed in the experiment shown in Figure 3.12. In the first instance, the anti-phosphotyrosine antibody FB2 was used to immunoprecipitate tyrosine phosphoproteins from quiescent and FcεR1 activated RBL2H3 cells. Analysis of these immunoprecipitations using anti-phosphotyrosine Western blot revealed a prominent 30kDa band in lysate from stimulated cells. This band was identified as the FcεR1β chain by use of the β-specific JRK-1 monoclonal antibody (Figure 3.12). By comparison with total lysate, this 30kDa band apparently represented a subpopulation of the total cellular β chain pool which was shifted to lower mobility in SDS-PAGE on KLH-DNP treatment, presumably reflecting its tyrosine phosphorylation. However, the 33kDa Grb2 binding tyrosine phosphoprotein was not immunoreactive with JRK-1 as shown in Figure 3.12. Therefore the p33 Grb2 binding protein described here cannot be the FcεR1β sub-unit.

In T cells a p36 protein has been described which is tyrosine phosphorylated in response to T cell antigen receptor triggering and associates with the Grb2 SH2 domain. The data in Figure 3.13 show a comparison of endogenous Grb2 SH2 domain binding proteins in RBL2H3 and Jurkat T cells. In the latter cells T cell receptor triggering via the CD3 ε chain induces the tyrosine phosphorylation of p36 and its binding to Grb2 SH2 domain. These data show that p33 and p36 clearly migrate differently on SDS-PAGE. However, p33 and p36 are clearly functionally analogous and are possibly homologous molecules. The difference in apparent molecular weight could be reconciled to the species difference between human Jurkat and Rat RBL2H3.

The data presented here are shown schematically in Figure 3.14. Two tyrosine phosphoproteins, Shc and p33, bind to endogenous Grb2 SH2 domains. Shc is constitutively tyrosine phosphorylated and bound to Grb2, whilst p33 is a substrate for FcεR1 activated PTKs.

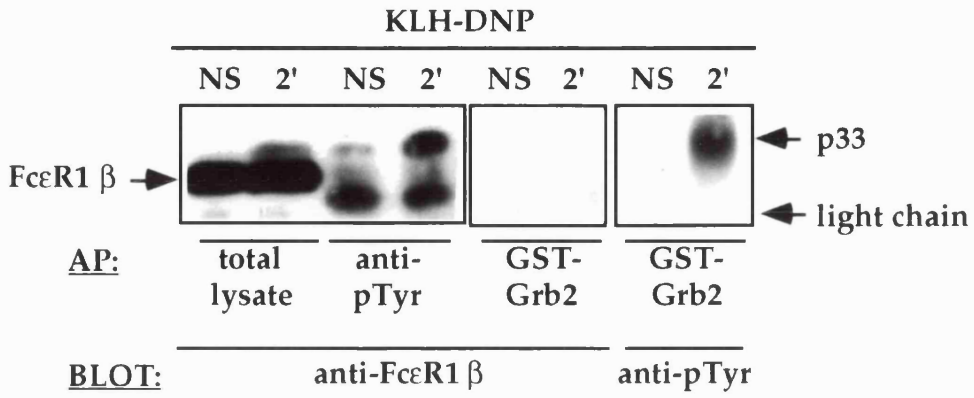


Figure 3.12. The Grb2 associated p33 tyrosine phosphoprotein in RBL2H3 is not the FcεR1 β chain. RBL2H3 were left unstimulated (NS) or primed with 1μg/ml IgE anti-DNP (1h, 37°C) before stimulation for 2 min at 37°C with 500ng/ml KLH-DNP. Proteins from the post-nuclear lysate of $1x10^6$ RBL2H3 per lane were acetone precipitated (total lysate). Affinity purifications (AP) from $1x10^7$ RBL2H3 per lane were carried out using either 5μg FB2 anti-phosphotyrosine antibody coupled to Protein G-sepharose or GST-Grb2 WT fusion protein. Proteins were resolved by 11% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in 5% non-fat milk before sequential probing with firstly polyclonal anti-FcεR1β at 2μg/ml followed by monoclonal anti-phosphotyrosine (4G10) (1μg/ml). Arrows mark the position of the FcεR1 β chain in total lysate (left), the position of immunoprecipitating antibody light chain in the anti-pTyr immunoprecipitations (right, bottom) and the p33 tyrosine phosphoprotein binding GST-Grb2 (right, top).

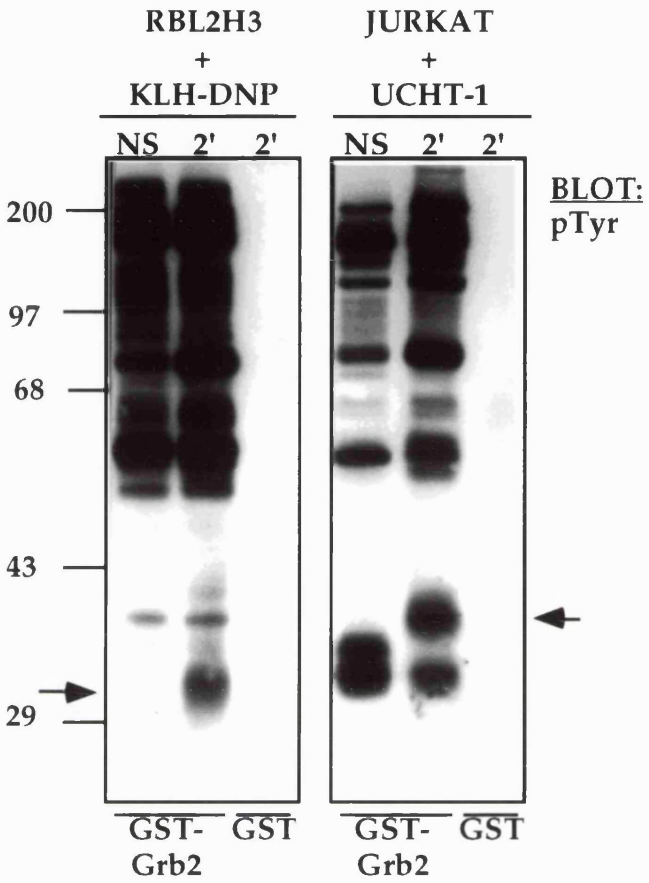


Figure 3.13. The RBL2H3 p33 protein and the T cell p36 protein are not equivalent. $1x10^7$ RBL2H3 per lane were left unstimulated (NS) or primed with 1µg/ml IgE anti-DNP (1h, 37°C) before stimulation for 15 min at 37°C with 500ng/ml KLH-DNP. $1x10^7$ Jurkat T cells per lane were left unstimulated (NS) or stimulated for 2 min at 37°C with 2µg/ml UCHT-1. Cells were lysed and affinity purifications were carried out as described in Materials and Methods. The affinity matrices used were the full length Grb2 fusion protein (GST-Grb2)or GST alone as a control. Proteins were resolved by 11% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in 5% non-fat milk before probing with monoclonal anti-phosphotyrosine (4G10) (1µg/ml). Arrows mark the positions of the RBL2H3 p33 (left) and Jurkat p36 (right) tyrosine phosphoproteins. Molecular weights are indicated in kDa.

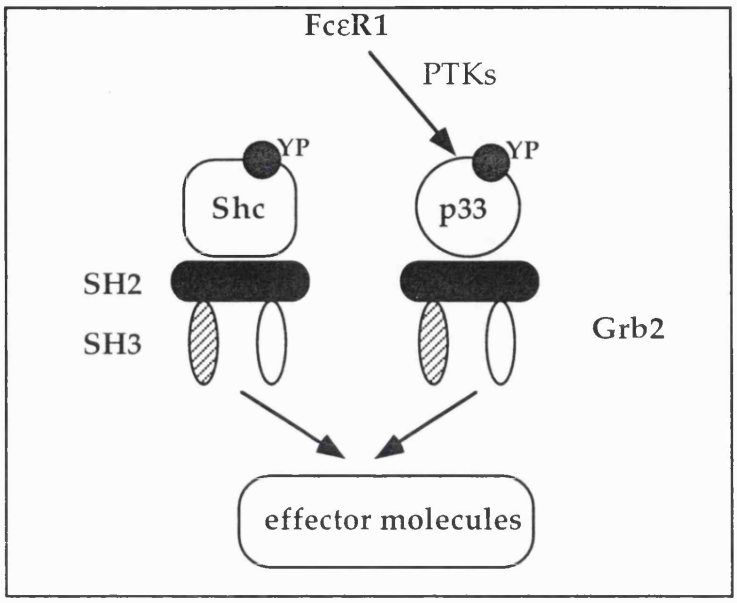


Figure 3.14. Summary of Grb2 SH2 domain interactions in RBL2H3. Sos; mammalian homologue of 'Son of Sevenless', SLP; SH2 containing lymphocyte protein. YP; phosphotyrosine, PTKs; protein tyrosine kinases.

3.10 Analysis of tyrosine phosphoproteins bound to the Grb2 SH3 domain in RBL2H3 reveals alternative Grb2 effectors.

Sos binds to the Grb2 SH3 domain in RBL2H3 and is a candidate effector molecule for Grb2. It is possible that Grb2 in fact has multiple effectors in RBL2H3. This would mean that Grb2 has pleiotropic functions in RBL2H3 by virtue of an ability to adapt to multiple downstream biochemical pathways. The experiments in Figure 3.8 have identified several substrates for FcεR1 associated kinases that could bind to the SH3 domains of Grb2 fusion proteins. Experiments to determine whether any of these proteins could interact with endogenous Grb2 complexes were therefore performed.

To determine whether any of the tyrosine phosphoproteins observed in GST-Grb2 affinity purifications are complexed to endogenous Grb2 SH3 domains, Western analysis using an anti-phosphotyrosine antibody was performed on the Grb2 complexes isolated by EGFR-Y1068 affinity purifications. These data are shown in Figure 3.15. Two basally tyrosine phosphorylated species were observed to bind to endogenous Grb2 SH3 as purified by EGFR-Y1068. It should be noted that there was a doublet of proteins binding non-specifically to control affinity purifications comprising Affigel-10 or GST beads alone. This doublet proved difficult to eliminate by pre-clearing. A 75kDa tyrosine phosphoprotein was observed in the endogenous Grb2 complexes isolated by EGFR-Y1068 affinity purifications from FcεR1 stimulated but not quiescent cells. This p75 species comigrated with that seen in GST-

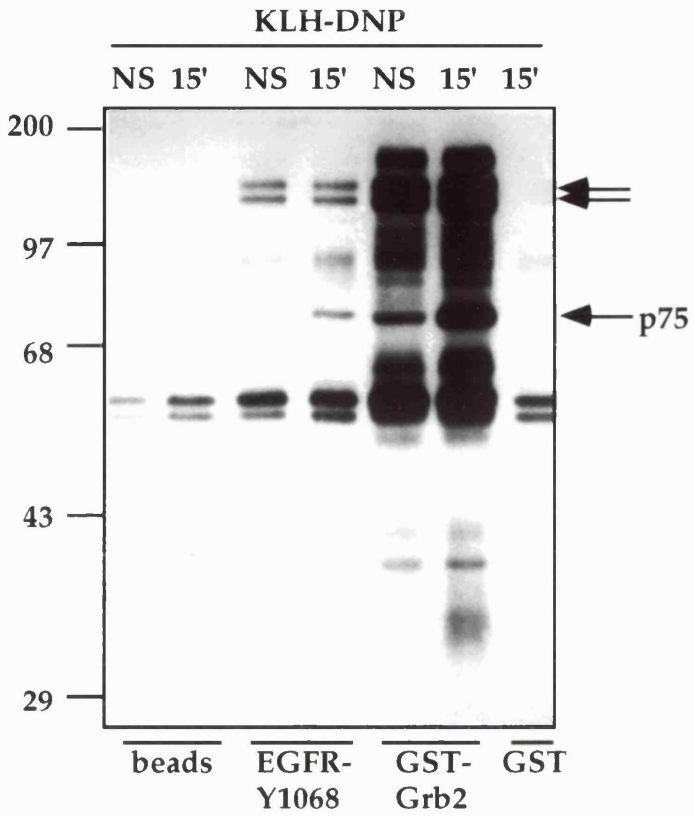


Figure 3.15. Tyrosine phosphoproteins of 75kDa and 110-120kDa associate with the Grb2 SH3 domain in RBL2H3. $1x10^7$ RBL2H3 per lane were left unstimulated (NS) or primed with 1µg/ml IgE anti-DNP (1h, 37°C) before stimulation for 15 min at 37°C with 500ng/ml KLH-DNP. Affinity purifications were carried out as described in Materials and Methods using affinity matrices of Affigel-10 beads alone (beads), the EGFR-Y1068 phosphopeptide or the GST-Grb2 WT fusion protein. Proteins were resolved by 11% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in 5% non-fat milk before probing with monoclonal anti-phosphotyrosine (4G10) (1µg/ml). Arrows mark the positions of the 75 and 110-120kDa tyrosine phosphoproteins in complex with the Grb2 SH3 domains. Molecular weights are indicated in kDa.

Grb2 fusion protein purification. By analogy with Sos, this p75 is therefore a candidate effector protein for Grb2.

The specificity of the purification of Sos and p75 by the EGFR-Y1068 tyrosine phosphopeptide/Grb2 complex was assessed. EGFR-Y1068 affinity purifications were performed in the absence and presence of a proline rich peptide derived from the Grb2 SH3 domain binding site of Grb2. This peptide, Sos-Pro, competitively prevented EGFR-Y1068 purification of either Grb2 associated Sos or p75. These data are shown in Figure 3.16.

EGFR-Y1068 peptide affinity purifications allow us to exclude the majority of the tyrosine phosphoproteins found complexed to the Grb2 SH3 domain fusion proteins from a physiological role since they are not found in association with endogenous Grb2. Grb2 SH3 domain binding proteins in RBL2H3 are therefore 1) a 75kDa tyrosine phosphoprotein from lysates of FcεR1 activated but not quiescent RBL2H3 cells and 2) Sos and 3) two proteins of 110 and 120kDa which are apparently equally tyrosine phosphorylated in quiescent and stimulated RBL2H3.

3.11 Further identification of Grb2 SH3 domain binding proteins in mast cells.

Sos is a major Grb2 SH3 domain binding protein and potential effector for Grb2 in mast cells. There are, however, other SH3 domain binding proteins which are candidates for Grb2 effectors in mast cells. The identity of the 75kDa SH3 binding tyrosine phosphoprotein was not clear at the time that this work was carried out. One substrate for FcεR1 activated tyrosine kinases which was recently identified is a 75kDa protein termed SPY or HS1. HS1 has an SH3 domain and proline rich sequences reminiscent of those in Sos which have high affinity for the Grb2 SH3 domain. Western blot analysis of Grb2 complexes isolated using the EGFR-Y068 peptide from either T cells or RBL2H3 showed that p75 is not HS1 (Dr. S. Ley, H. Turner., unpublished observations).

A 75-76kDa protein analogous to the p75 Grb2 binding phosphoprotein was identified in the T cell by Reif *et al.* (1996). This protein has subsequently been cloned and sequenced from the T cell and given the name SLP-76 (SH2 domain containing protein of 76kDa). Therefore, when a polyclonal antibody to SLP-76 became available to us, an obvious question was whether the mast cell p75 protein was in fact SLP-76. The SLP-76 antibody was a generous gift of Dr David Williams, Roche PLC, UK.

The data in Figure 3.17 show an affinity purification of the p75 tyrosine phosphoprotein in the Grb2 complexes isolated from RBL2H3 cells using the EGFR-Y1068 peptide. The top panel shows Western analysis with the anti-SLP-76 antibody, whilst the bottom panel shows a re-probing of the same membrane with anti-phosphotyrosine. These data show that the p75 mast cell protein which binds

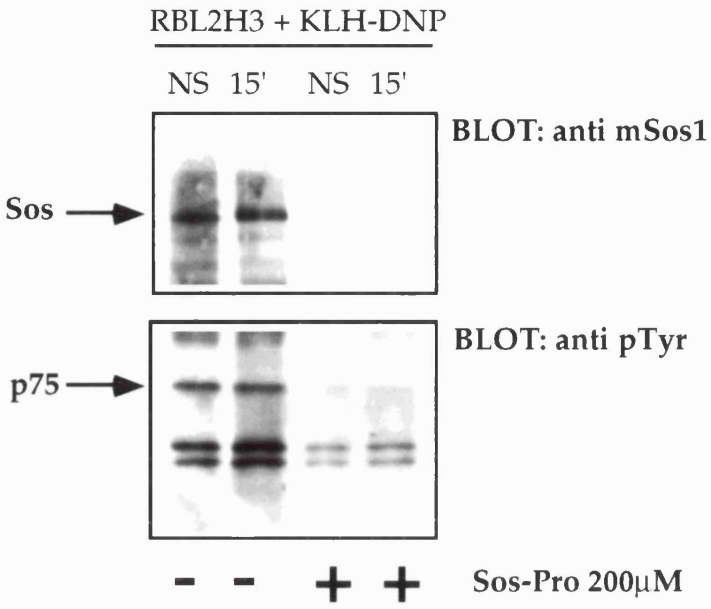


Figure 3.16. A proline rich peptide derived from Sos effectively competes the Grb2/Sos and Grb2/p75 interactions. $1x10^7$ RBL2H3 per lane were left unstimulated (NS) or primed with 1μg/ml IgE anti-DNP (1h, 37°C) before stimulation for 15 min at 37°C with 500ng/ml KLH-DNP. Affinity purifications were carried out as described in Materials and Methods using the EGFR-Y1068 affinity matrix in the absence or presence as indicated of 200μM Sos-Pro peptide. Proteins were resolved by 11% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in 5% non-fat milk before probing with the indicated antibodies.

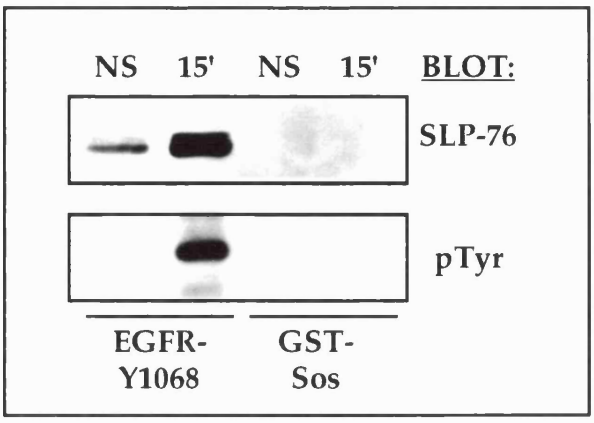


Figure 3.17. The 75kDa tyrosine phosphoprotein associated with the Grb2 SH3 domains in RBL2H3 is SLP-76. $1x10^7$ RBL2H3 per lane were left unstimulated (NS) or primed with 1µg/ml IgE anti-DNP (1h, 37°C) before stimulation for 15 min at 37°C with 500ng/ml KLH-DNP. Affinity purifications were carried out as described in Materials and Methods using affinity matrices of the EGFR-Y1068 phosphopeptide or the GST-Sos fusion protein. Proteins were resolved by 11% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in 5% non-fat milk before probing with firstly polyclonal anti-SLP-76 (2µg/ml) followed by stripping of the membrane and reprobing with monoclonal anti-phosphotyrosine (4G10) (1µg/ml).

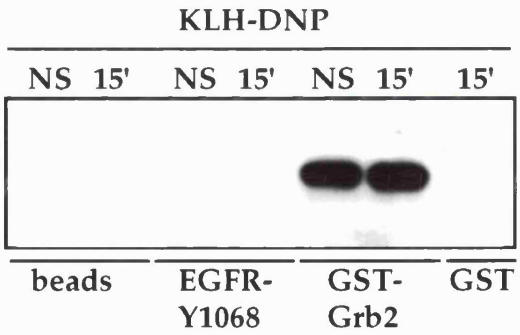


Figure 3.18. The 120kDa tyrosine phosphoprotein associated with the Grb2 SH3 domains in RBL2H3 is not the *cbl* oncogene product. $1x10^7$ RBL2H3 per lane were left unstimulated (NS) or primed with 1µg/ml IgE anti-DNP (1h, 37°C) before stimulation for 15 min at 37°C with 500ng/ml KLH-DNP. Affinity purifications were carried out as described in Materials and Methods using affinity matrices of Affigel-10 beads alone (beads), the EGFR-Y1068 phosphopeptide, the GST-Grb2 WT fusion protein or GST alone. Proteins were resolved by 11% SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in 5% non-fat milk before probing with monoclonal anti-cbl (1µg/ml).

endogenous Grb2 SH3 domains is recognised by an antibody to the SLP-76 protein cloned from T cells. There is a basal association of SLP-76 with Grb2 SH3 domains in RBL2H3 which is enhanced on FcεR1 crosslinking. This is clearly an SH3 domain mediated interaction since SLP-76 is not identified in Grb2 complexes associated with the GST-Sos fusion protein.

In addition to Sos and SLP-76, a constitutively tyrosine phosphorylated 120kDa protein was observed to bind to the SH3 domains of endogenous Grb2 in mast cells. Recent data in the T cell system has shown that p120cbl can bind to a GST-fusion protein of the amino-terminal Grb2 SH3 domain. p120cbl is a pluripotent adapter molecule which is a substrate for T cell receptor associated tyrosine kinases. This protein is a candidate for the 120kDa Grb2 SH3 domain binding protein observed in the RBL2H3. The data in Figure 3.18 show a Western analysis of Grb2 fusion protein and EGFR-Y1068 affinity purifications with a monoclonal antibody to p120cbl. p120cbl was detected in affinity purifications performed with the wild-type GST-Grb2. However, p120cbl is clearly not present in a complex with the endogenous Grb2 molecule in RBL2H3, as purified by the EGFR-Y1068 peptide.

To summarise, as shown in Figure 3.19, endogenous Grb2 SH3 domains in RBL2H3 bind two identified species, the Sos GEF and SLP-76. The latter is inducibly tyrosine phosphorylated in response to FcεR1 crosslinking. Both species are bound to the Grb2 SH3 domains constitutively. Two unidentified tyrosine phosphoproteins of 110 and 120 kDa also bind the Grb2 SH3 domain in RBL2H3.

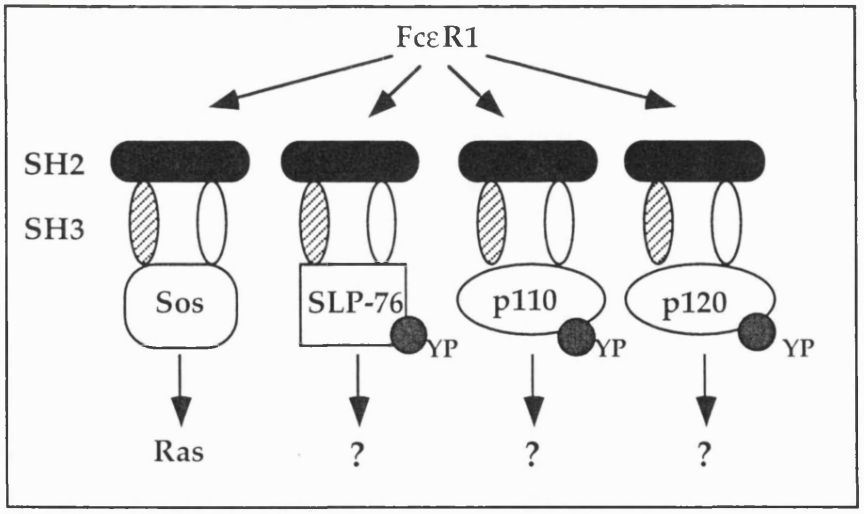


Figure 3.19. Summary of Grb2 SH3 domain interactions in RBL2H3. Sos; mammalian homologue of 'Son of Sevenless', SLP; SH2 containing lymphocyte protein. YP; phosphotyrosine.

3.12 Information transmission across the p33-Grb2 complex.

The nature of information transmission across the Grb2 complex was the focus of considerable debate at this time. The data presented so far have identified the components of Grb2 complexes in RBL2H3. Possible routes for signal transduction across these Grb2 complexes are shown in Figure 3.20. Information may be transmitted from the receptor associated PTKs via p33-Grb2 to effector molecules in a purely linear fashion (case A). However, it is equally possible that effector molecules can receive information from receptor associated PTKs independently of p33 (case B).

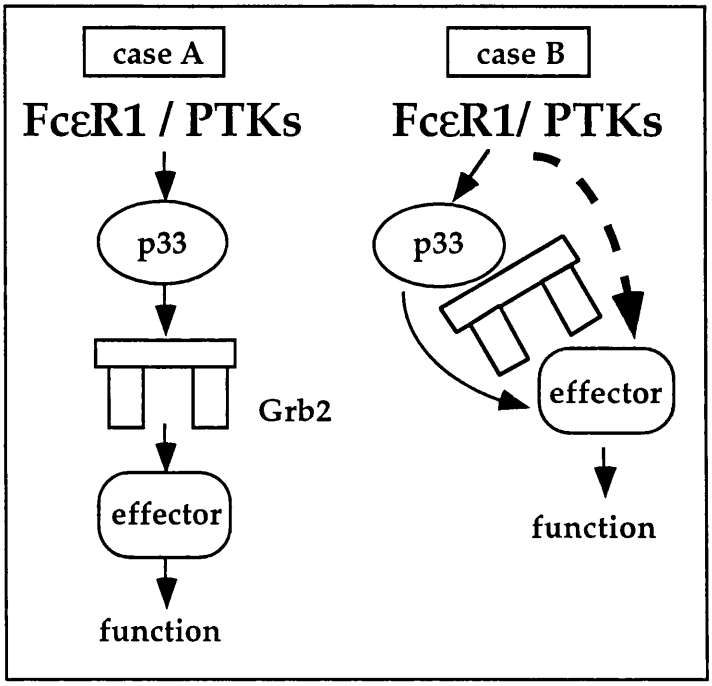


Figure 3.20. Routes for the transmisssion of information across the Grb2 complex. A PTK derived signal may be transmitted in a linear fashion. Alternatively, effector molecules may be substrates for FcεR1 associated PTKs independently of p33 activity.

In order to investigate this point, an assay was developed for Grb2 complex analysis in RBL2H3 permeabilised using the pore-forming bacterial toxin, Streptolysin O (SLO). Streptolysin-O binds and polymerises cholesterol found in the outer leaflet of the plasma membrane into ring-like lesions. These pores are stable, homogeneous and are approximately 12nm in diameter. SLO pores permit the entry into cells of membrane impermeant reagents such as nucleotides, peptides and hydrophilic proteins. Since the SLO target is cholesterol, membranes other than the plasma lemma are unaffected. The aim of the assay was to introduce into SLO permeabilised RBL2H3 reagents which would selectively disrupt interactions of the Grb2 SH2 and SH3 domains. Affinity purifications of Grb2 binding proteins from these cells would then

be performed, asking if any features of FcεR1 regulation of Grb2 complexes such as tyrosine phosphorylation of individual components remained intact.

Since it is well established that SLO pores permit the entry of peptides, an obvious candidate for a reagent to disrupt the p33/Grb2 interaction was the EGFR-Y1068 peptide. This peptide should clearly be able to act as a competitor for endogenous Grb2 SH2 binding proteins when applied as free peptide to permeabilised cells. Since the application of phosphatase inhibitor to permeabilised cells is not advisable, it was necessary to synthesise a non-hydrolysable phospho-analogue of the EGFR-Y1068 (EGFR-Y1068^{A}) peptide for use in this assay. The tyrosine analogue used was phenyl methyl phosphate.

Control experiments were performed to optimise the conditions for SLO permeabilisation of RBL2H3. With careful temperature regulation and use of the indicated buffer, it was found that 85-89% of RBL2H3 became permeabilised (as assessed by trypan blue positivity under light microscope) within 2 min of exposure to 0.4 i.u/ml SLO. This type of efficiency was important in order to avoid contaminating signals from intact cells.

The data in Figure 3.21 show that the application of 250μM EGFR-Y1068^{A} peptide as a bathing solution to SLO treated cells competes detectable p33-Grb2 interaction. In comparison with control cells no tyrosine phosphorylated p33 is seen in GST-Sos affinity purifications from SLO/EGFR-Y1068^{A} treated cells. However, in EGFR-Y1068^{A} treated cells there is still significant inducible tyrosine phosphorylation of SLP-76, as purified using the GST-Grb2NSH3 fusion protein affinity matrix. These data indicate that disruption of the p33/Grb2 interaction 1) does not prevent SLP-76 interaction with the Grb2 SH3 domains and 2) does not adversely affect the FcεR1 regulated tyrosine phosphorylation of SLP-76.

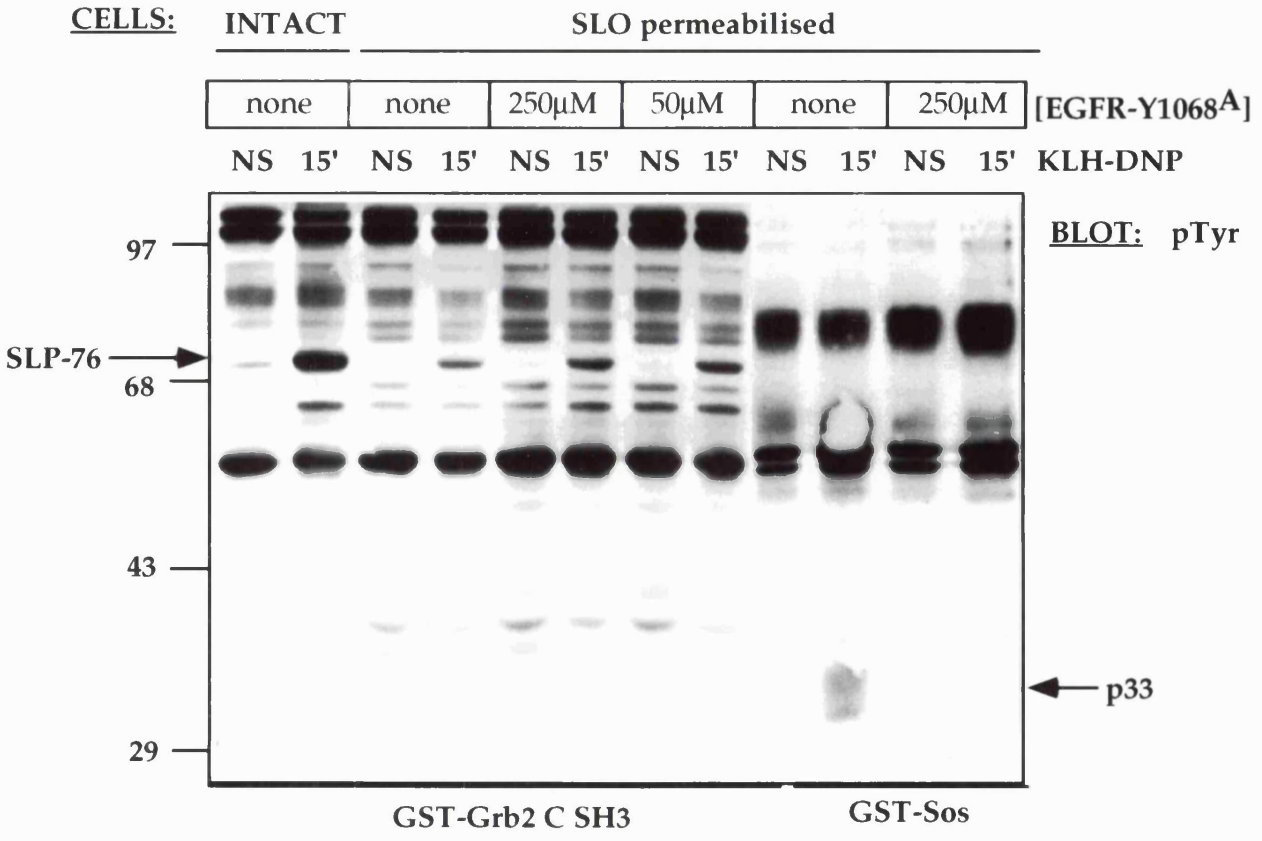


Figure 3.21. SLP-76 tyrosine phosphorylation is not dependent on the p33/Grb2 interaction in RBL2H3. RBL2H3 were primed as described and left intact or permeabilised as described in Materials and Methods using 0.4i.u./ml SLO/point in DMEM/10% FCS. SLO start solution contained 250μM or 50μM EGFR-Y1068A peptide where indicated. Intact or permeabilised cells were left unstimulated (NS) or IgE primed and stimulated with 500ng/ml KLH-DNP for 15 min. Cell lysates were affinity purified for 2h with the indicated GST fusion protein as described in Materials and Methods. Samples were resolved under reducing conditions by 11% SDS-PAGE before transfer to PVDF membrane and anti-phosphotyrosine Western analysis using 1μg/ml 4G10. The positions of tyrosine phosphorylated SLP-76 and p33 are indicated by arrows.

3.13 DISCUSSION

The data presented in this chapter have shown that regulation of protein complexes formed around the adapter molecule Grb2 follows FcεR1 stimulation in mast cells. The interactions of Grb2 characterised here are summarised in Figure 3.22.

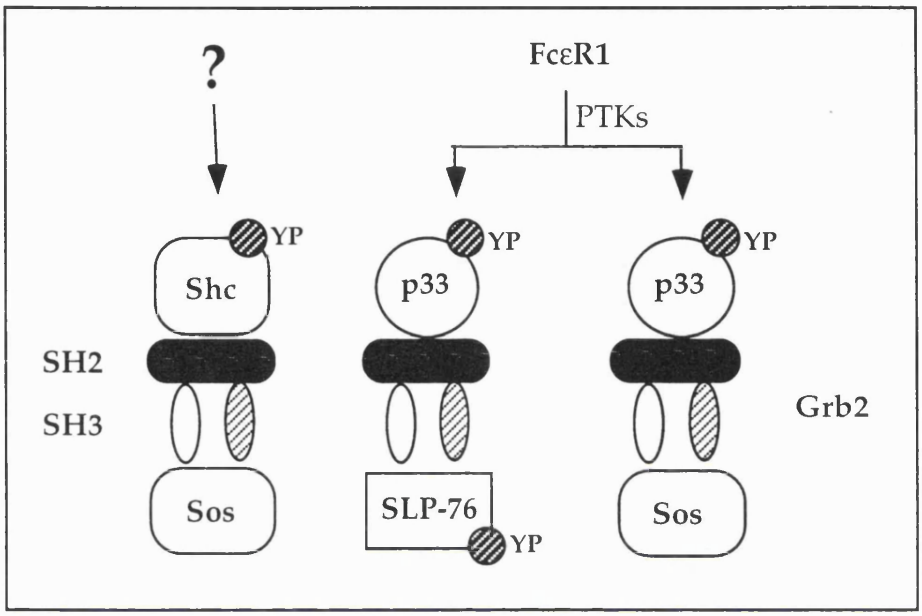


Figure 3.22. Summary of Grb2 protein complexes found in RBL2H3. YP; phosphotyrosine, PTK, protein tyrosine kinase.

3.13 A 33kDa tyrosine phosphoprotein recruits Grb2/Sos to the membrane in RBL2H3.

Recruitment of the Sos GEF to the membrane brings Sos into proximity with its substrate, Ras. As described above, there are a number of published mechanisms by which this is accomplished in antigen receptor regulated cells. Sos is in a complex with the Grb2 SH3 domains in RBL2H3. The data presented in this chapter have shown that the major FcεR1 regulated mechanism for Grb2/Sos membrane recruitment in RBL2H3 is via a novel adapter protein p33. The p33 protein is a substrate for FcεR1 associated PTKs and is not the FcεR1β chain. Fusion proteins of Grb2 will bind to the FcεR1 subunit, but there is no evidence for an interaction of FcεR1β and endogenous Grb2 in RBL2H3. There is an analogous adapter protein in T cells, the p36 molecule which links TCR associated PTKs and Grb2 complexes. The RBL2H3 p33 and T cell p36 molecules are functionally analogous but are not the same protein. It is interesting to note that data from the Weiss laboratory places p36 as an adapter binding to the SH2 domain of Phospholipase C-γ1 in T cells (Sieh *et al.*, 1994). By analogy, p33 may therefore play a role in adapting to multiple signalling pathways initiated by FcεR1 crosslinking.

p33 is not the sole binding partner of the Grb2 SH2 domains in RBL2H3. The Shc adapter binds endogenous Grb2 SH2 domains. This interaction is constitutive because Shc is basally tyrosine phosphorylated in unstimulated RBL2H3. Neither the tyrosine phosphorylation of Shc nor its Grb2 association are FcεR1 regulated. This is analogous to the T cell situation, where Shc/Grb2/Sos complexes are present but the predominant complex in T cells is p36/Grb2/Sos (Buday *et al.*, 1994). In contrast, ligation of the B cell antigen receptor induces both tyrosine phosphorylation of Shc and its association with Grb2.

Shc/Grb2/Sos complexes are present in RBL2H3 but are not FcεR1 regulated. It is clear that antigen derived signals are only one type of information received by a mast cell in its physiological context. Cytokine, growth factors and context derived signals will also be present. It is reasonable to expect that receptors one or more of these factors, which are known to activate Ras, use Shc/Grb2/Sos complexes to achieve this. For example, Ras is activated by the IL-3 receptor in primary mast cells and the use of Shc to adapt to Grb2-Sos complexes by various cytokine receptors including the IL-3 receptor is also documented. There have been a number of recent reports detailing the involvement of Shc-Grb2 complexes and hence Ras activation in signalling following ligation of integrins such as the laminin receptor. A logical extension of this model would be to predict that transformed RBL2H3, which are growth factor independent and have constitutive adhesive interactions, could contain basally tyrosine phosphorylated Shc and hence pre-formed Shc-Grb2-Sos complexes.

The laboratory of Kinet has recently demonstrated that RBL2H3 which have been deprived of serum can exhibit a low level of FcεR1-induced Shc tyrosine phosphorylation; the authors argue therefore that Shc cannot be discounted as part of the mechanism by which the FcεR1 activates Ras (Jabril-Cuenod *et al.*, 1996). The authors of this paper do not attempt to detect other Grb2 SH2 domain binding proteins such as p33. However, a recent analysis in (Hendricks-Taylor *et al.*, 1997) clearly demonstrates that tyrosine phosphoproteins 33-36kDa associate with Grb2 SH2 domains. These authors were unable to detect any Shc binding even to the Grb2 SH2 domain fusion protein.

3.14. Sos and SLP-76 are effector molecules for FcεR1 regulated Grb2 complexes in RBL2H3.

Sos is complexed to the Grb2 SH3 domains in RBL2H3. This linkage between the FcεR1 and Sos provides a mechanism for FcεR1 regulation of the guanine nucleotide binding status of the Ras GTPase. Sos promotes GTP loading and hence activation of Ras. In other cell systems, Ras.GTP is known to activate diverse effector pathways and control diverse functional outcomes. The regulation of transcription

factors by Ras effector pathways in RBL2H3 is a major focus of this thesis and will be discussed more extensively later.

By analogy with Sos, SLP-76 is an effector molecule for Grb2 in RBL2H3. SLP-76 was cloned from T cells where it is also found in complex with Grb2 SH3 domains. Since the recognition of the protein in T cells it has been the object of intensive study in order to define its function in that system. TCR stimulation of T cells results in the induction of multiple genes, including that for IL-2. IL-2 acts in an autocrine fashion on T cells, promoting the G1/S transition and hence T cell clonal expansion. Nuclear Factor of Activated T cells (NFAT) transcription factors are critical regulators of IL-2 gene induction for T cells (see Chapters 5-7 for more detail). In the T cell NFAT activity is a target for Ras, Rac-1 and calcium signals (Genot *et al.*, 1996) NFAT is an important marker of T cell activation and commitment to cytokine gene inductions. Wu *et al* observed that overexpression of SLP-76 in Jurkat T cells markedly potentiated TCR induction of NFAT and IL-2 production (Wu *et al.*, 1996). Mutation of the SLP-76 SH2 domain partly blocked this enhancement, suggesting that the SLP-76 SH2 interaction with a tyrosine phosphorylated target protein may be mechanistically important. NFAT may be an important transcription factor in mast cells. There are NFAT binding sites in the IL-4 promoter among others (Rao *et al.*, 1997).

There is a considerable body of work on the biochemical interactions of SLP-76. First, the SLP-76 kinase has been identified in the T cell as ZAP-70 and in the RBL2H3 as Syk. ZAP-70 and Syk are tandem SH2 containing kinases which are recruited to ITAMs in activated antigen receptor aggregates. In T cells SLP-76 is clearly a pleiotropic effector, binding to as yet uncharacterised tyrosine phosphoproteins of 62 and 120kDa as well as the 'SLP associated protein' SLAP. SLP-76 also interacts in T cells with the protein product of the *vav* proto-oncogene (Musci *et al.*, 1997). This interaction may have important functional consequences.

Vav-1 is tyrosine phosphorylated in response to both TCR and FcεR 1 stimulation (Katzav *et al.*, 1994; Margolis *et al.*, 1992). Vav-1 contains multiple structural motifs found in intracellular signalling proteins; PH, cysteine rich, SH2 and SH3 domains are present. Vav-1 also has a Dbl homology domain characteristic of guanine nucleotide exchange factors (GEFs) for Ras family GTPases (Khosravi-Far *et al.*, 1994). It has recently been confirmed that Vav-1 is a GEF for the Rac-1 and CDC42 GTPases (Crespo *et al.*, 1997). Vav-1 apparently has no exchange activity for Ras. The interaction between Vav-1 and SLP-76 is mediated by the Vav-1 SH2 domain binding to tyrosine phosphorylated SLP-76. This interaction is therefore a possible connection between the FcεR1 regulated Grb2 complex and Rac-1/CDC42 GTPases. Grb2 complexes may adapt to two distinct GTPase targets in RBL2H3 1) Ras, via Grb2 SH3 recruitment of Sos and 2) Rac-1/CDC42 via Grb2 SH3 recruitment

of Vav-1. This model is illustrated in Figure 3.23. The functional significance of Rac-1/CDC42 activation is not known in T cells or mast cells. However, these GTPases in other systems have published roles in regulation of actin cytoskeleton rearrangement. Moreover, it is clear that Rac-1, like Ras, plays a role in the induction of NFAT transcriptional activity in T cells.

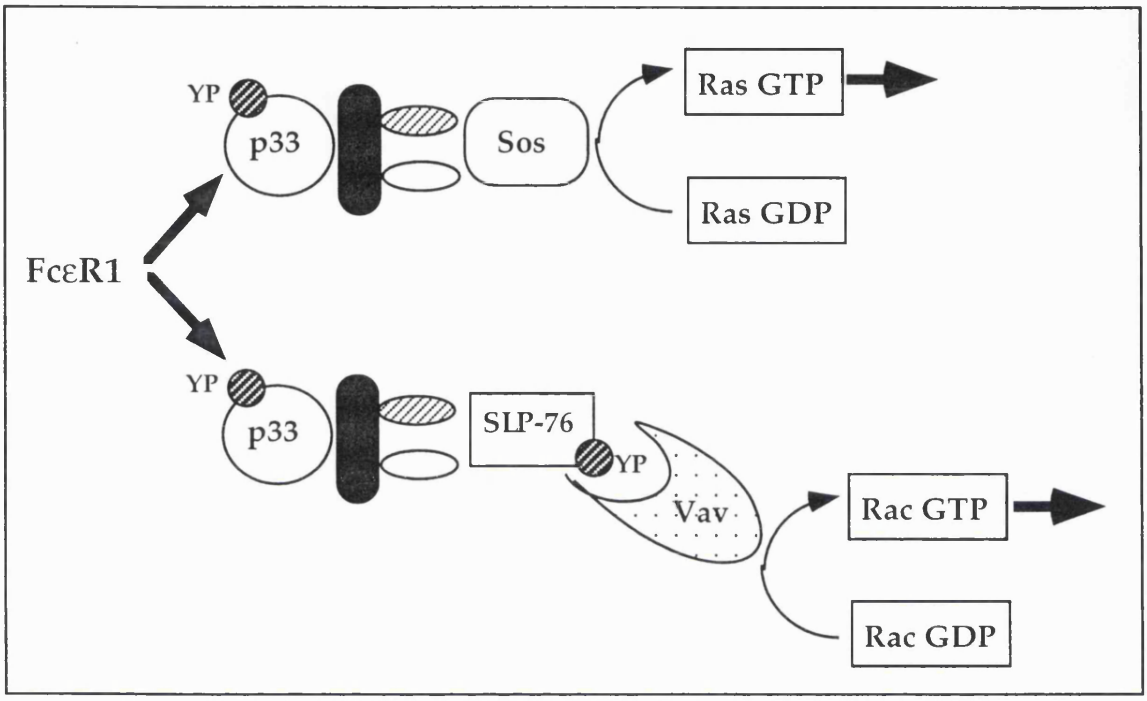


Figure 3.23. Grb2 cassettes postulated to link FcεR1 associated PTKs with Ras and Rho family GTPases. GTPases are membrane localised by virtue of their prenylation. Grb2 complexes recruit GEFs directly or indirectly into proximity with their target GTPases. GEFs then promote GTP loading and hence activation of the GTPase, which mediates diverse downstream effects.

3.15. Information transmission across the Grb2 complex in RBL2H3.

One of the characteristics of multiprotein complexes is that they may integrate inputs from multiple regulatory signalling molecules. Protein complexes nucleated by the Grb2 adapter are no exception to this. It is not clear how many upstream inputs from regulatory molecules there are converging on the Grb2 complex. It is also not clear whether ligation of the SH2 domain actually alters affinity/avidity of the SH3 domains for effector proteins. A simple model would be that a single upstream input is required, derived in this case from FcεR1 regulated PTKs. This input is actively transduced by conformational change in Grb2, altering the nature of the SH3 domain interaction. A 1994 study (Cussac *et al.*, 1994) sought to resolve whether phosphotyrosine/SH2 domain ligation alters affinity of SH3 for proline-rich motifs. The data show that binding of tyrosine phosphopeptides to SH2 does not affect the affinity of Grb2 SH3 for Sos-Pro. However, this does not necessarily exclude a Grb2

induced conformational change in Sos from potentiating exchange activity without altering affinity of the basic interaction. Reif *et al* (1992) showed that proteins complexed to the Grb2 SH3 domain could interfere with SH2 mediated interactions. A recent study also showed that Grb2/Sos complexes have an approximately 4-fold higher affinity for the EGFR-Y1068 peptide than Grb2 alone (Chook *et al.*, 1996). Hence the interaction of Grb2 with an 'effector' may influence the binding of the 'upstream' SH2 domain to tyrosine phosphorylated targets.

The data presented in the last section of Chapter 2 asked the question of whether the tyrosine phosphorylation of SLP-76 is reliant on the p33/Grb2 interaction. In permeabilised cells where there was no detectable p33/Grb2 interaction the tyrosine phosphorylation of SLP-76 was equivalent to that in control cells. These data suggest that SLP-76 comes into contact with its kinase, Syk, independently of the p33/Grb2 interaction. Therefore, FcεR1 regulation of SLP-76 does not fit into a simple model where tyrosine phosphorylated p33 recruits Grb2/SLP-76 and SLP-76 is then brought into contact with Syk. Instead, it appears that there are two distinct routes for information from FcεR1 regulated PTKs to impinge upon components of the Grb2 complex. This opens the question of what the purpose of the p33/Grb2 interaction is in the p33/Grb2/SLP-76 complex. The experiment does not address the question of how productive the Grb2/SLP-76 interaction is in the presence or absence of p33/Grb2. It is possible that 1) there is a quantitative difference in the number/sites of tyrosine phosphorylation of SLP-76 in the presence of p33/Grb2 or 2) that SLP-76 tyrosine phosphorylation is a docking event and another level of regulation, perhaps mediated by a p33/Grb2 conformational change transmitted to SLP-76, is required for optimal activity of SLP-76 and a productive interaction with a putative effector such as Vav-1.

What is the functional significance for mast cell activation of the FcεR1 regulation of Grb2 complexes? Following the work presented in this chapter, a number of approaches were taken to identify roles for Grb2 complexes in aspects of mast cells function such as 1) secretion of allergic mediators and 2) gene induction. The approaches relied on the disruption of Grb2 complex formation. The first assay which was developed involved the introduction of competitor peptides into permeabilised cells, followed by measurement of FcεR1 induced hexoseaminidase release. These peptides were shown to specifically compete the p33/SH2 interaction of Grb2 and to block the Grb2/Sos interaction. However, the application of this assay was limited since 1) no reagent was found that competed the Grb2/SLP-76 interaction and hence no judgement could be made about the role of this major Grb2 binding protein in secretion. 2) it became clear from the biochemical data generated in parallel with this assay (see Figure 3.21) that proteins in complex with the SH2 and SH3

domains of Grb2 can be directly regulated by FcεR1. Hence no judgement can be made about the importance of the Grb2 SH3/effector interactions in the absence of p33/Grb2 binding when those effectors may be targeted by the FcεR1 in a p33 independent manner.

A second approach involved the use of expression vectors carrying wild-type and mutant forms of Grb2 in transient transfection experiments. Transient transfec tion efficiencies in RBL2H3 are of the order of 30%. Therefore, in order for the effect of Grb2 mutants on responses such as secretion to be assayed, it was necessary to sort the transfected cells and perform secretion assays selectively on that population. Cell sorting was attempted by FACS and immuno-magnetic bead separation. Neither technique provided sufficient numbers of viable RBL2H3 for subsequent assay of secretory responses. Without the development of an actual reporter gene assay for secretion (i.e. transient transfection of constructs encoding an exogenous tagged protein which is packaged and secreted by RBL2H3 in response to FcεR1 stimulation), it was clear that the role of Grb2 complexes in the control of secretion was refractory to analysis.

Secretory responses are only one facet of FcεR1 induced mast cell activation. *De novo* gene induction of immediate early and cytokine genes contributes to the initiation and maintenanace of an inflammation. Newly synthesised chemokines and cytokines form chemotactic gradients for leukocyte infiltration and contribute to the activation of these effector cells. It is clearly of interest to study the regulatory mechanisms for *de novo* gene induction by the FcεR1.

The data presented in Chapter 3 have identified that regulation of Grb2/Sos complexes is a consequence of FcεR1 stimulation in the mast cell line RBL2H3. As the guanine nucleotide exchange factor for Ras, Sos initiates diverse functional events mediated by signalling cascades downstream of Ras. FcεR1 stimulation of Ras GTP loading has been reported (Jabril-Cuenod *et al.*, 1996) and it seems likely that the Ras GTPase may be an important regulator of mast cell function. Therefore, from this point experiments were directed towards determining the functional significance of Ras regulation by the FcεR1. The data to be presented in Chapter 4 detail experiments which identify a transcription factor target for Ras mediated signals in RBL2H3.

CHAPTER FOUR

The transcription factor Elk-1 is a target for Ras signalling pathways in the RBL2H3 cell line.

4.1 INTRODUCTION

The FcεR1 regulates protein complexes formed around the adapter molecule Grb2. Grb2 provides a linkage to, and membrane recruitment mechanism for, the guanine nucleotide exchange factor Sos, the mammalian homologue of the Drosophila 'Son of sevenless' protein. When brought to the membrane, Sos activity promotes GTP loading and hence activation of the Ras GTPase. There had previously been no study of the roles of Ras itself and Ras family proteins in the FcεR1/mast cell system. Therefore, once the Grb2/Sos activatory mechanism for Ras was identified in this system, it was important to then identify downstream targets for Ras effector pathways. The data in this chapter will detail the identification of the Elk-1 transcription factor as a nuclear target for Ras signals in the FcεR1/mast cell system. Moreover, the effector pathway by which Ras activates Elk-1 is identified.

4.1.1 Immediate early gene induction and regulation.

Ligation of cell surface receptors for antigen on immune cells results in a large number of activation events. On the basis of certain thresholds for stimulation, an antigen receptor stimulated cell will make commitment decisions towards survival, growth, differentiation and expression of function. Whilst a short cellular response time may be critical in these processes, it is not efficient for cells to continually contain and maintain a full complement of the proteins necessary to respond to potential stimuli. A solution to this complex issue lies in the existence of the 'immediate early' gene class. Immediate early gene expression does not require *de novo* protein synthesis for initiation of transcription. These are genes whose protein products are detectable in the cytoplasm with very rapid stimulation kinetics. For example, the prototypical gene of this class is that coding for the c-*fos* protein. c-*fos* is transcribed within 10 min of T cell antigen receptor stimulation of peripheral blood T cells. This observation is typical of c-*fos* induction across a number of systems.

The promoter of the c-*fos* gene has been the object of intensive investigation. Its regulation is a paradigm for the type of signalling network which links transmembrane (growth factor) receptors to the activation of intracellular proto-oncogene products, reviewed in (Hill and Treisman, 1995b; Treisman, 1994;

Treisman, 1996). The c-*fos* promoter contains a number of upstream regulatory elements including the cytokine regulated sis-inducible element (SIE) and the Serum Response Element (SRE) (Hill and Treisman, 1995a). The SRE is sufficient to confer growth factor, serum and phorbol ester sensitivity to the the c-*fos* gene. At the SRE a ternary protein complex forms which is comprised of a dimer of the Serum Response Factor (SRF) and a member of the Ternary Complex Factor (TCF) family. Only TCF bound in the context of SRF has affinity for DNA or is sufficient to mediate gene induction. TCF proteins are a subgroup of the large ETS-domain family of transcription factors, originally characterised on the basis of sequence homology to the *ets-1* proto-oncogene product. TCFs are ETS-domain proteins which can form productive interactions with SRF. Three TCFs are described in humans, Elk-1 (Rao *et al.*, 1898), SAP-1 (Dalton and Treisman, 1994) and SAP-2 (Price *et al.*, 1995b). These proteins have the general domain structure (Figure 4.1) which comprises an N-terminal DNA-binding ETS box (consensus sequence ACCGGAAGTR), the SRF-interacting 'B' box and a C-terminal regulatory region with a number of sites for MAP kinase phosphorylation.

A current model for the formation of the ternary complex is also shown in Figure 4.1. This model, proposed by Richard Treisman, draws on domain deletion experiments and biochemical evidence of *in vivo* interactions. The 'B' box is identical to the regions required for ternary complex formation with SRF, but deletion or addition of residues in the inter ETS-B region does not affect this complex formation. Substitution of the ETS box alone is enough to confer altered specificity of DNA binding, assigning DNA binding function to this region (Hill and Treisman, 1995a). In addition, a direct interaction between the 'B' box and SRF has been demonstrated, this is apparently the sole contact point between TCFs and the SRF dimer.

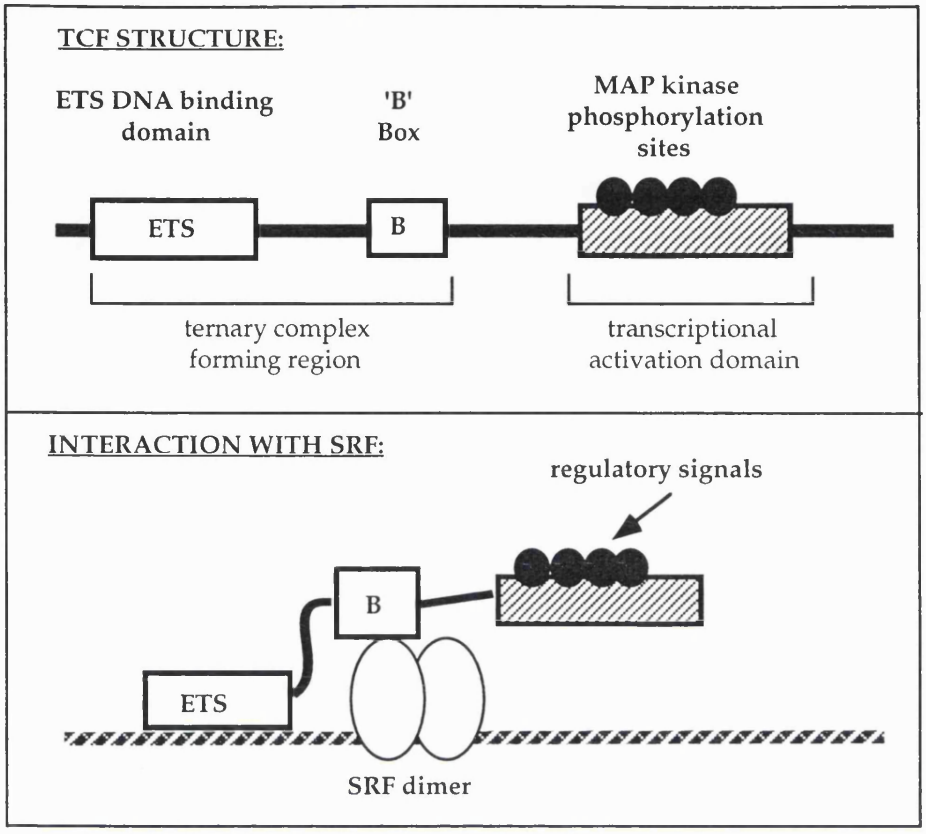


Figure 4.1. General domain structure of TCF family members such as Elk-1 and model for productive interaction with the SRF. After Treisman (1996).

4.1.2 Elk-1 is a ternary complex factor that is expressed in immune cells and is a target for MAP kinases.

TCFs such as Elk-1 are a subgroup of the extensive ETS homology family of transcription factors. Whilst other ETS proteins have many sites of regulation of important immune genes (eg. cytokine and chemokine promoters, MHC components, immunoglobulin genes, integrin and immunotoxin genes), TCFs have only been shown to regulate the c-*fos* and c-*egr-1* promoters in immune cells. It seems likely that there are other targets for TCFs in immune cells which are yet to be characterised.

Elk-1 is a 63kDa TCF protein which is expressed in a variety of tissues including cells of hematopoietic origin (Bassuk and Leiden, 1997). The consensus DNA sequence for Elk-1 binding is CAGGAT. As shown above, the C-terminal region of Elk-1 contains a number of conserved potential sites for phosphorylation by MAP kinase family members. The *in vitro* phosphorylation of these sites was shown in 1992 (Gille *et al.*, 1992). Later, Elk-1 was shown to be a substrate for ERK1 and ERK2 nuclear kinases (Gille *et al.*, 1995a; Marais *et al.*, 1993) Phosphorylation of Elk-1 at two sites, Ser 383 and Ser 389, enhances formation of the ternary complex and hence growth factor induced c-*fos* promoter activity. Site-directed mutagenesis of

these sites impairs c-*fos* responsiveness. Subsequent data have added an additional level of complexity in Elk-1 regulation in that it can be a substrate for MAP kinases other than ERKs. Studies using protein synthesis inhibitors as differential activators of MAP and Stress-Activated kinase revealed that in HeLa cells, Elk-1 can integrate signals from ERKs and Jun kinase (p54 SAPKβ) (Zinck *et al.*, 1995). However, it is not entirely clear whether physiological stimuli of the different kinase cascades reproduce these results within a single cell system. It is clear that TCF regulation is accomplished by diverse mechanisms according to the stimulus applied. This situation is summarised in Figure 4.2.

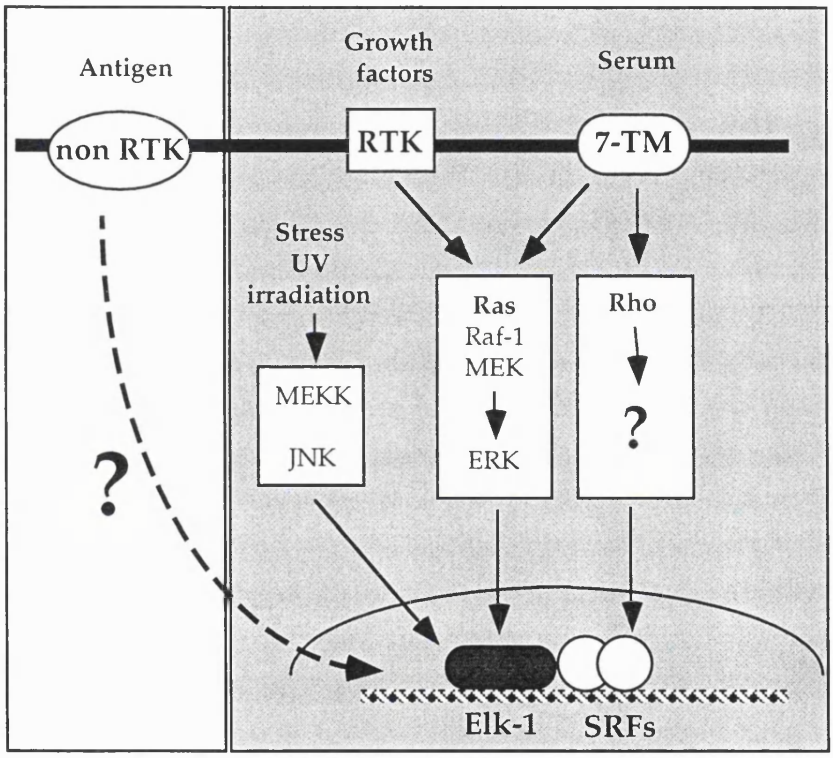


Figure 4.2. Elk-1 is a target for multiple signalling pathways. Ras family GTPases transduce signals from Growth factor Receptor Tyrosine Kinases (RTKs) and Seven Transmembrane domain receptors (7-TM) to the Serum Response Element comprising binding sites for the Ternary Complex Factor Elk-1 and a dimer of Serum Response Factors (SRFs). It is not known how non-tyrosine kinase receptors for antigen in immune cells may regulate Elk-1.

Elk-1 has also been reported to be a target for the classical second messenger cyclic adenosine monophosphate (cAMP). cAMP is known to promote growth and differentiation in a variety of cell types, these effects are in part mediated by cAMP induction of gene transcription. A report (Vossler *et al.*, 1997) places Elk-1 as a target for cAMP dependent signals in a neuronal cell line PC12. The data show that in this system another Ras family GTPase, Rap-1, activated by the cAMP dependent Protein

kinase A (PKA), regulates ERK and Elk-1. This activation is apparently reliant on the function of the Raf-1 isoform B-Raf. It is clear that Elk-1 can be activated by multiple mechanisms. Disparate upstream signals ranging from tyrosine kinase/adapter molecule cascades to classical second messengers all activate Elk-1.

4.1.3 Ras regulates multiple effector pathways which in turn control the activation of downstream Ras targets.

Ras signals regulate Elk-1 in a variety of systems. There is diversity at the level of the kinase which directly acts upon Elk-1, depending on the nature of the initiating signal input. Elk-1 regulation by different MAP kinases could reflect 1) that multiple parallel GTPase/kinase cascades (eg derived from Ras and terminating in ERK, derived from Rac and terminating in JNK) can regulate Elk-1 or 2) that Elk-1 is a target for different effector pathways which diverge downstream of Ras itself.

There is now much evidence that Ras and other GTPases regulate multiple signalling pathways. Molecules that interact with Ras.GTP (Ras effectors) and GTP bound forms of other GTPases are being identified in increasing numbers, by techniques varying from yeast-two-hybrid screening to direct 'pull-down' assays of GTP dependent interactions. Moreover, analysis of the GTP dependent conformational changes in Ras molecules allowed rational deign of mutants with altered effector selectivity. Two regions of Ras alter their conformation in a nucleotide dependent manner. The switch II region is N-terminal and highly mobile. The Switch I region is composed of residues 30-40, point mutational analyses of this region have shown that certain mutations ablate the ability of oncogenic (Val 12) Ras to signal but do not affect GTP binding (Marshall, 1996). These effector mutations are altering key residues that are the specificity determinants of Ras.GTP interaction with a particular effector molecule. Ras interacts with diverse targets in evolutionarily divergent organisms. Genetic data from yeast *S. pombe* and *S. cerevisiae* indicate that there are multiple targets for Ras in each organism (Chang *et al.*, 1994). There is also evidence for multiple Ras effectors in higher organisms (White *et al.*, 1995).

Characterised Ras effectors are shown in Figure 4.3 and reviewed more fully in (Marshall, 1996). These are proteins which have been demonstrated to interact with Ras.GTP. The relative importance or even existence of these interactions across a spectrum of cell types is not well understood for all effectors. The best characterised case is interaction with Raf-1 and subsequent induction of the MEK/ERK signalling cascade. In addtion to the direct effector protein interactions shown below, there is a class of indirect transducers of Ras.GTP dependent signals. For example, there is considerable evidence that Ras family GTPases may act in cascades. Thus Rac-1 and Rho can act downstream of Ras in regulation of growth factor induced rearrangements

of the fibroblast actin cytoskeleton. It is also established that oncogenic transformation by Ras can be Rac-1 dependent and that by Rac-1 can be Rho dependent.

	EFFECTOR	ACTIVITY
Direct interactions	**Raf-1**	Ser/Thr kinase. Directs induction of the MEK/ERK cascade.
	Ral-GDS	Guanine nucleotide dissociation inhibitor of the Ral GTPase. Promotes GTP/GDP exchange. Role in oncogenic transformation by Ras.
	NF-1	Neurofibromin-1. GTPase activating protein for Ras
	p120 GAP	GTPase activating protein for Ras
	PI 3-kinase	Phosphatidy inositol 3'hydroxyl kinase. Possible roles in directing Ras and Rac mediated actin cytoskeleton rearrangement.
Indirect interactions	**Rac**	Rho family GTPase. Rac-1 may transduce Ras signals to the actin cytoskeleton or towards oncogenic transformation
	MEKK-1	MAP KKK (ie. Raf-1 equivalent) in the signaling cascade terminating in Jun kinase activation.

Figure 4.3. Ras effectors identified in mammalian cells. These proteins provide potential for a single upstream input to Ras to regulate diverse cellular outcomes. For references see (Marshall, 1996) and citations therein.

4.1.4 Antigen receptors induce immediate early genes.

The regulation of the c-*fos* gene by the FcεR1 is established (Lewin *et al.*, 1996; Stephan *et al.*, 1997). The FcεR1 is also thought to induce c-*jun* activity, but regulation of any of the numerous other immediate early genes has not been examined in this system. The protein product of the c-*fos* proto-oncogene, Fos, is an important transcription factor in leukocytes. It is a partner in the AP-1 transcription factor

complex which is known to participate in the regulation of a variety of immune response genes. AP-1 homo- or hetero-dimers are required for the optimal activity of NFAT (Nuclear Factor of Activated T cells). NFAT/AP-1 transcription factor complexes control induction of multiple immune response genes including those for IL-2, IL-4, IL-3, GM-CSF and the Fas- and CD40-ligand molecules (Rao *et al.*, 1997). The FcεR1 regulation of NFAT is a major topic of this thesis and will be returned to in detail later.

Elk-1 is highly expressed in B- and T- lymphocytes (Bassuk and Leiden, 1997) Regulation of Elk-1 transcriptional activity by the T cell antigen receptor has been described (Genot *et al.*, 1996). Whilst the Elk-1 regulation of the c-*fos* promoter is well established, no hematopoietic specific gene targets for Elk-1 have yet been identified. The co-operative role of Fos and NFAT family members does however suggest that regulation of Elk-1 may be an important consequence of antigen receptor stimulation. Moreover, Elk-1 may well have non-SRE sites of action in promoters other than that of the c-*fos* proto-oncogene. Taken together the importance of Elk-1 as a gene regulator in many systems and its clear regulation by GTPase/kinase cascades suggest that this may be an interesting object of study in the RBL2H3 system. In order to look at Elk-1, a transient transfection approach was employed using a CAT (Chloramphenicol acetyl transferase) reporter gene system established by the laboratory of Dr Richard Treisman at ICRF (Marais *et al.*, 1993).

Elk-1 is clearly a target for Ras in fibroblasts. There has previously been no examination of targets for Ras mediated signals in RBL2H3 or of the regulation of immediate early gene induction. The identification of Ras.GTP targets in the FcεR1 regulated RBL2H3 is an objective of the work presented in this thesis. Therefore the following questions were asked:

1) Is Elk-1 a target for Ras family GTPase signals in FcεR1 stimulated RBL2H3?

2) Is Elk-1 regulated by a specific MAPK Ser/Thr kinase cascade in Rbl2H3?

4.2 RESULTS

4.3 The LexAOPtk.CAT/LexA Elk-1 reporter gene system for measurement of Elk-1 transactivation.

This assay is a sensitive technique for the measurement of Elk-1 transcriptional activity. Induction of reporter gene activity by a transcription factor usually requires two distinct events, both of which may be open to regulation by upstream signals and which are together termed transcativation. These events are DNA binding of the factor and subsequent recruitment of the basal transcriptional machinery. The LexA-Elk-1/Lex-OPtk CAT reporter system measures the signalling requirements for activation of a DNA-bound Elk-1 fusion protein (Marais *et al.*, 1993; Price *et al.*, 1995b) LexA OP.tk CAT comprises two copies of the Lex A reporter. This system involves the transfection of a reporter gene construct of a bacterially derived LexA binding site upstream of the CAT reporter gene under a thymidine kinase minimal promoter (LexA OP.tk CAT). LexA OP.tk CAT and a plasmid encoding a fusion protein of the LexA DNA binding domain and Elk-1 C-terminal region under a constitutive promoter are cotransfected (pEF-NLexA Elk-1C). The LexA region of the fusion protein binds constitutively to its site on the reporter gene construct. Activatory signals phosphorylate the Elk-1C region of the fusion protein, leading to recruitment of transcriptional machinery and CAT gene induction. This process is shown schematically in Figure 4.4.

Initial experiments were carried out to establish the optimal conditions for use of this assay in the RBL2H3. The data in Figure 4.5 show an experiment designed to identify the optimal ratio of fusion protein to reporter gene plasmids in this co-transfection system. Cells were stimulated with the phorbol ester PdBu, an activator of Protein Kinase C. These data identify that 12μg LexA OPtk.CAT to 6μg LexA Elk-1 fusion protein plasmids is optimal, giving a 12 fold induction of reporter gene activity over background in this experiment. Higher DNA concentrations did not increase this level of induction.

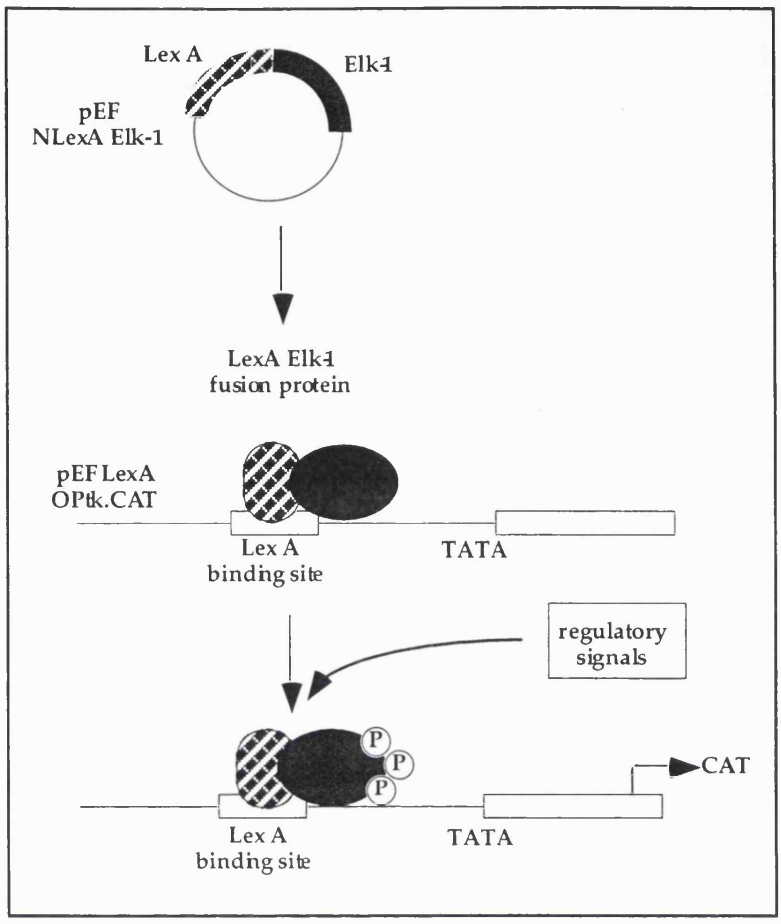


Figure 4.4. The LexAOPtk.CAT/LexA Elk-1 reporter gene system for measurement of Elk-1 transactivation. Two plasmids are co-transfected, pEF NLexA Elk-1 encodes a fusion protein under a constitutive promoter. Binding of the LexA protein to its site within the reporter gene construct is constitutive. Regulatory signals phosphorylate Elk-1. Recruitment of basal transcriptional machinery and CAT gene induction follow.

The data in Figure 4.6 show that in cells expressing LexA OPtk.CAT alone stimulation of RBL2H3 with PdBu was not able to induce reporter gene activity. In cells coexpressing LexA OPtk.CAT and the LexA Elk-1 fusion protein, PdBu typically induced >10 fold induction in LexA OPtk.CAT reporter gene activity. As would be expected, there is virtually no background CAT activity from this assay in the mammalian RBL2H3, since CAT activation is dependent on binding of the bacterially derived LexA fusion protein to its target sequence. Given the data from these experiments, the LexA OPtk.CAT/LexA Elk-1 reporter system was used to examine mechanisms of Elk-1 transactivation in FcεR1 stimulated RBL2H3.

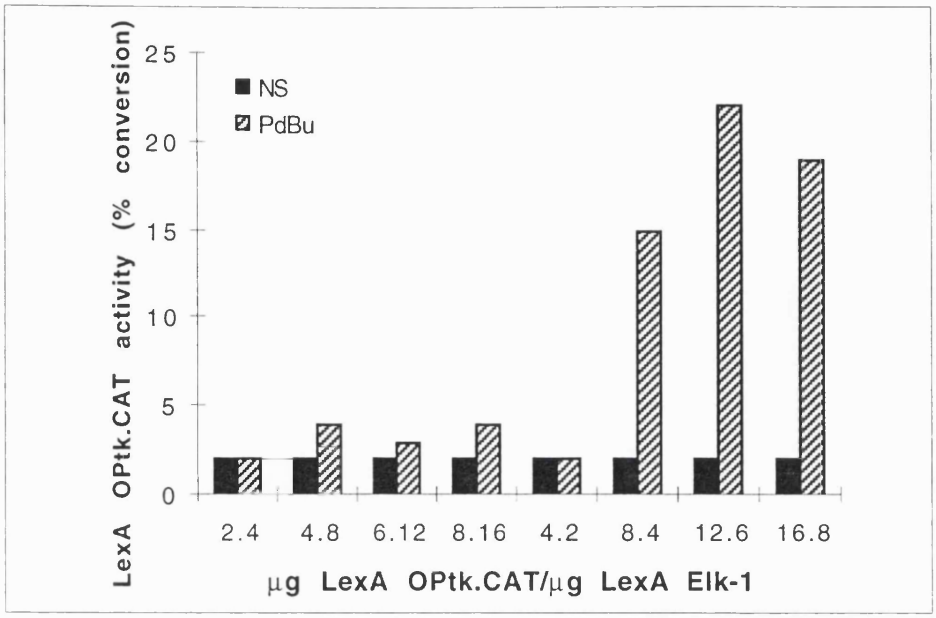


Figure 4.5. Optimisation of DNA ratios for Elk-1 transactivation assay in RBL2H3. $1x10^7$ RBL2H3 were transfected per point with the indicated microgram amounts of CAT reporter and fusion protein DNAs. Cells were recovered for 6h and stimulated for 14h with 50ng/ml PdBu or vehicle control before harvesting and assay as described in Materials and Methods. Data are representative of two experiments.

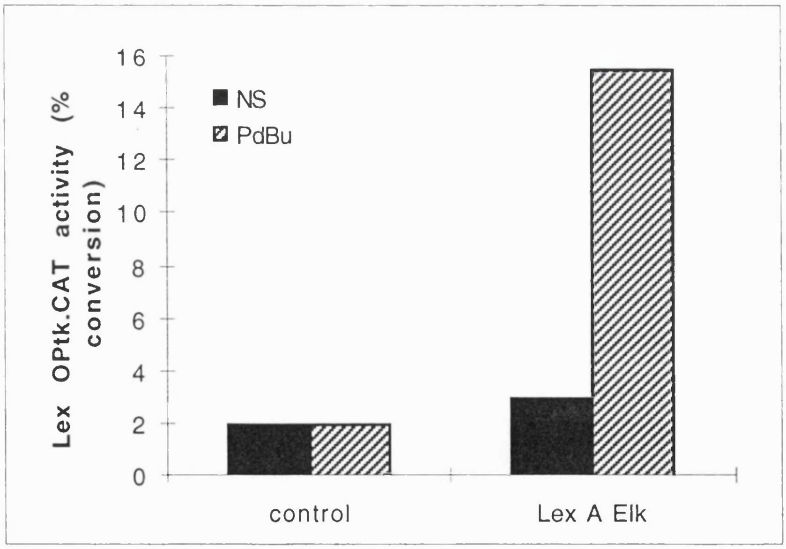


Figure 4.6. Stimulation of LexA OPtk.CAT activity is dependent on the presence of co-transfected LexA Elk-1 fusion protein. $1x10^7$ RBL2H3 were electroporated with 12µg LexA OPtk.CAT reporter alone (control) or in combination with 6µg LexA Elk-1. Cells were recovered for 6h then stimulated with 50ng/ml PdBu or vehicle control for 14h before assay as described. Data are representative of two experiments.

4.4 Elk-1 transactivation in RBL2H3 is induced by the FcεR1 and phorbol esters but not by the action of calcium ionophore.

The effect of various pharmacological stimuli on LexA OPtk.CAT activity was assessed. The phorbol ester PdBu stimulates Elk-1 transactivation in RBL2H3 in a dose-dependent manner (Figure 4.7). Figure 4.7 also shows that elevation of intracellular free calcium levels using the ionophore Ionomycin does not stimulate Elk-1 transactivation and that Ionomycin does not enhance the stimulatory affect of PdBu. These data strongly suggest that Elk-1 transactivation in RBLH3 is not a calcium regulated event. However, the data do suggest that Elk-1 may be a target for one of the multiple PKC signalling pathways induced by the action of PdBu.

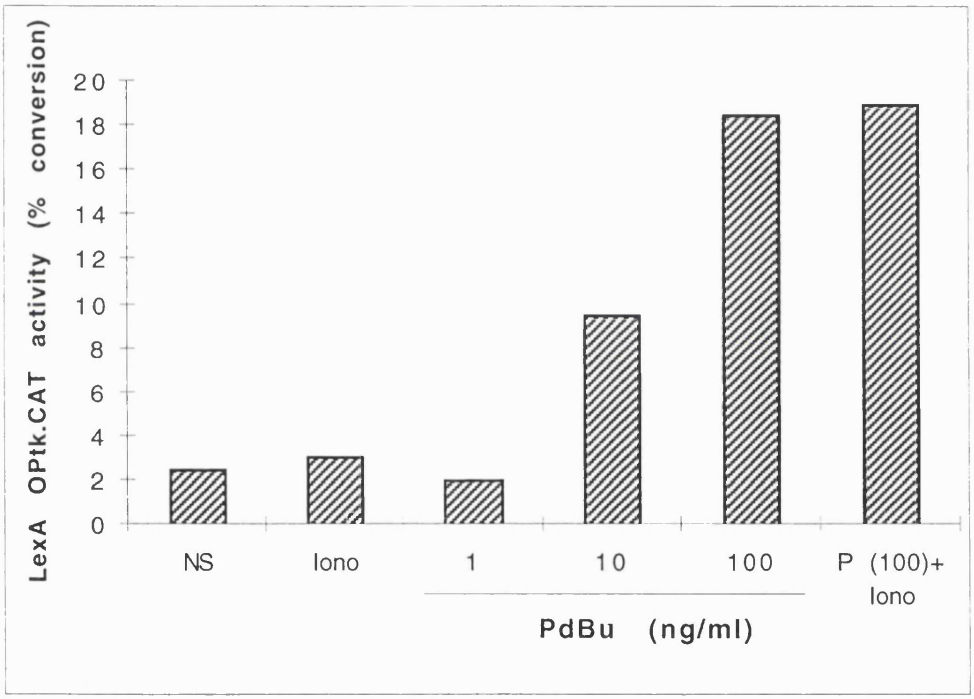


Figure 4.7. Phorbol esters but not ionomycin stimulate Elk-1 transactivation in RBL2H3. $1x10^7$ RBL2H3 were electroporated with 12μg LexA OPtk.CAT reporter in combination with 6μg LexA Elk-1. Cells were recovered for 6h then stimulated with the indicated concentration of 500ng/ml Ionomycin or 50ng/ml PdBu alone or in combination as indicated. NS is vehicle control. Stimulations were for 14h before assay as described. Data are representative of 3 experiments.

The aim of the work presented in this Chapter is to examine mechanisms of Elk-1 regulation by the FcεR1 in the mast cell line RBL2H3. The data in Figure 4.8 establish that Elk-1 is regulated by the FcεR1 in a manner dose-dependent on the concentration of crosslinking KLH-DNP antigen applied. Across a number of experiments, the maximum Elk-1 activation was achieved by 400-500ng/ml KLH-DNP, while the EC_{50} ([KLH-DNP] for 50% maximal stimulation) was approximately 100ng/ml. These data indicate that Elk-1 activity is strongly induced by FcεR1

stimulation of RBL2H3. Experiments were therefore directed towards analysis of the signalling mechanism used by the FcεR1 to target this transcription factor.

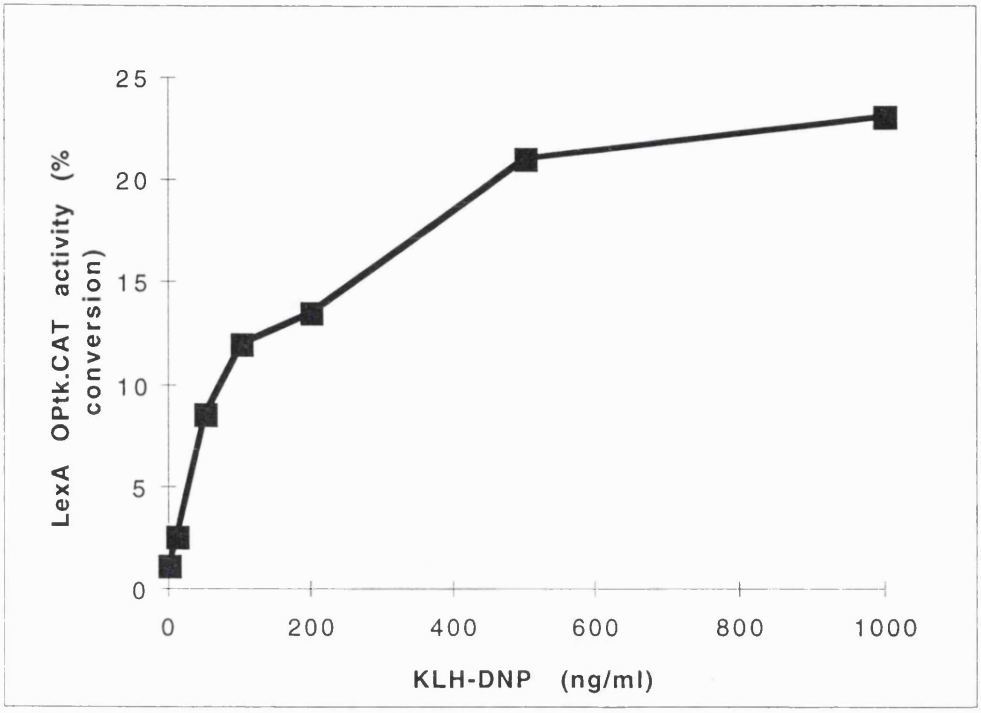


Figure 4.8. Antigenic crosslinking of the FcεR1 induces Elk-1 transactivation in a manner dependent on antigen dose. $1x10^7$ RBL2H3 were electroporated with 12μg LexA OPtk.CAT reporter in combination with 6μg LexA Elk-1. Cells were recovered for 6h then stimulated with the indicated concentration of KLH-DNP multivalent antigen for 14h before assay as described. Data are representative of 3 experiments.

4.5 Induction of Elk-1 transactivation by the antigenic crosslinking of the FcεR1 is not dependent on Protein Kinase C.

FcεR1 ligation is known to activate Protein kinase C isoforms, and previous studies have established that PKC mediated signals are important for the induction of exocytosis. More relevantly, studies of Fos mRNA synthesis in permeabilised RBL2H3 purport to have shown that reconstitution of permeabilised cells with purified PKC β2 or δ can induce Fos mRNA production. PKC activation is therefore a candidate mechanism for FcεR1 control of Elk-1.

Structural analogues of the microbial metabolite Staurosporine have been shown to be potent and selective inhibitors of the PKC isoenzyme family. Their activity has been established both using purified recombinant enzyme and in cellular systems. An inhibitor of this class, the bis-indoylmaleimide compound Ro-318425, was made available by Dr David Williams, Roche Research Centre, UK. The data in Figure 4.9 show that a 500nM dose of Ro-318425 inhibits the phorbol ester induction

of Elk-1 transactivation in mast cells by 75%. In contrast, FcεR1 induction of Elk-1 transactivation was independent of the presence of Ro-318425.

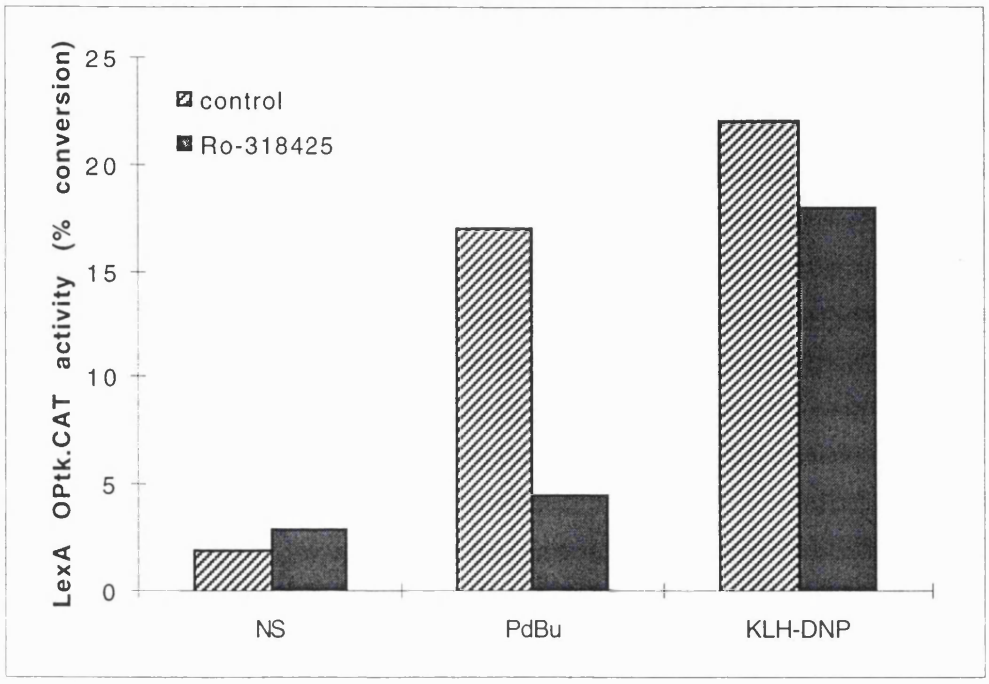


Figure 4.9. The Protein kinase C inhibitor Ro-318425 inhibits PdBu but not FcεR1 induced Elk-1 transactivation in RBL2H3. $1x10^7$ RBL2H3 were electroporated with 12μg LexA OPtk.CAT reporter in combination with 6μg LexA Elk-1. Cells were recovered for 6h then pre-incubated for 30 min with vehicle or 500nM Ro-318425. Cells were left unstimulated (NS) or treated with either 50ng/ml PdBu or 250ng/ml KLH-DNP as indicated. Stimulation was for 14h before assay as described. Data are representative of 3 experiments.

The data in Figures 4.8 indicate that signals derived from the FcεR1 regulate the transcriptional activity of Elk-1 in RBL2H3. The insensitivity of FcεR1 stimulation of Elk-1 activity to the PKC inhibitor Ro-318425 suggests that PKC-mediated signals are not essential for FcεR1 regulation of Elk-1. Raising of intracellular calcium levels by the ionophore Ionomycin did not induce Elk-1 activity. Therefore candidate non-calcium/PKC pathways for FcεR1 regulation of Elk-1 were investigated.

4.6 Wild-type and mutant forms of the Grb2 adapter protein could not be used to assess the contribution of Grb2 complexes to Elk-1 activation in RBL2H3.

The data presented in Chapter 3 detail that regulation of protein complexes formed around the Grb2 adapter molecule is a major consequence of FcεR1 crosslinking in RBL2H3. It is of interest to attempt to assign a function to Grb2 in RBL2H3, by assessing the consequences of disruption of Grb2 complexes for a functional response. Towards this end, expression vectors bearing wild-type and mutant forms of Grb2 were used in co-transfection experiments with the reporter gene system for Elk-1 transactivation. These constructs were made by Dr Carol Beadling in the Lymphocyte Activation Laboratory and comprised 1) wild-type Grb2, 2) Grb2 with a loss of function mutation in the SH2 domain and 3) Grb2 with loss of function point mutations in both SH3 domains. The latter proteins might be expected to act as a dominant inhibitory mutants, either by binding upstream activators to Grb2 which cannot interact with SH3 domain binding effector molecules such as Sos or by sequestering membrane proximal activators of Grb2 into non-productive interactions.

For assessment of the effects of these mutant Grb2 proteins on Elk-1 activation, it is a pre-requisite that the expression of the mutant protein can be confirmed. Initial experiments sought to confirm by Western blot that co-transfection of the expression plasmids for wild-type (WT) and mutant Grb2 resulted in significant expression of the exogenous protein. The pEF-bos expression vectors carrying the Grb2 mutants were designed to modify the protein with a myc tag, resulting in a lower mobility in SDS-PAGE for the exogenous compared to the endogenous Grb2. A number of techincal problems were encountered. Large amounts of plasmid DNA (30-40μg) were required per 10^7 cells in order to obtain expression of any of these constructs visible by Western blot. This instantly precluded the possibility of using these plasmids at this level in co-transfection for reporter gene assays. Including the reporter system, the total amount of DNA used would need to be 50-60μg, an amount toxic to cells at which transfection efficiencies drop to unworkable levels. Moreover, between plasmids, expression levels were not equivalent. There were 2-3 fold differences in expression of the wild-type versus mutant Grb2 molecules. Taken together, these results meant that the role of Grb2 complexes as candidate non-calcium/PKC signals in Elk-1 activation could not be explored.

4.7. Activated mutants of the Ras family GTPases Ras, Rac-1 but not RhoA induce Elk-1 transactivation in RBL2H3.

Extensive data from other cell systems has established that Elk-1 can be a target for MAP kinases which are terminal components of kinase cascades initiated by GTPases of the Ras family. Thus in RBL2H3, Elk-1 could be a target for a similar

GTPase/kinase cascade. To examine this point activated mutants of Ras, Rac-1 and Rho were used in cotransfection with the LexA OPtk.CAT/LexA Elk-1 reporter gene system. These activated mutants are rendered insensitive to the action of GAP proteins and are thus locked in a GTP-bound, active state. The data in Figure 4.10 show that expression of activated (V12) mutants of Ras and Rac-1 but not activated V14 RhoA potently induce Elk-1 transactivation in the absence of other stimulation. Moreover, V12 Rac-1 enhances the receptor mediated induction of Elk-1 transactivation observed in RBL2H3.

Expression of V14Rho in cells transfected with pEF V14 Rho was confirmed by Western blot analyses (data not shown). In experiments run in parallel with the Elk-1 assays above, a functional control was also used for the efficacy of the V14 Rho construct. In Jurkat T cells the activity of a CAT reporter gene driven by the Serum response Element (SRE) is strongly regulated by a Rho dependent mechanism. The data in Figure 4.11 show the results of an SRE-CAT assay performed in Jurkat T cells stimulated via the TCR/CD3 complex using the UCHT-1 antibody. In these cells, V14 Rho strongly induced SRE-CAT activity in the absence of other stimulation. These data suggest that the V14 Rho mutant used here is clearly capable of driving Rho dependent signalling pathways. This control experiment could not be performed in RBL2H3 since neither pharmacological stimuli, antigen nor activated GTPase mutants induced SRE-CAT activity in these cells.

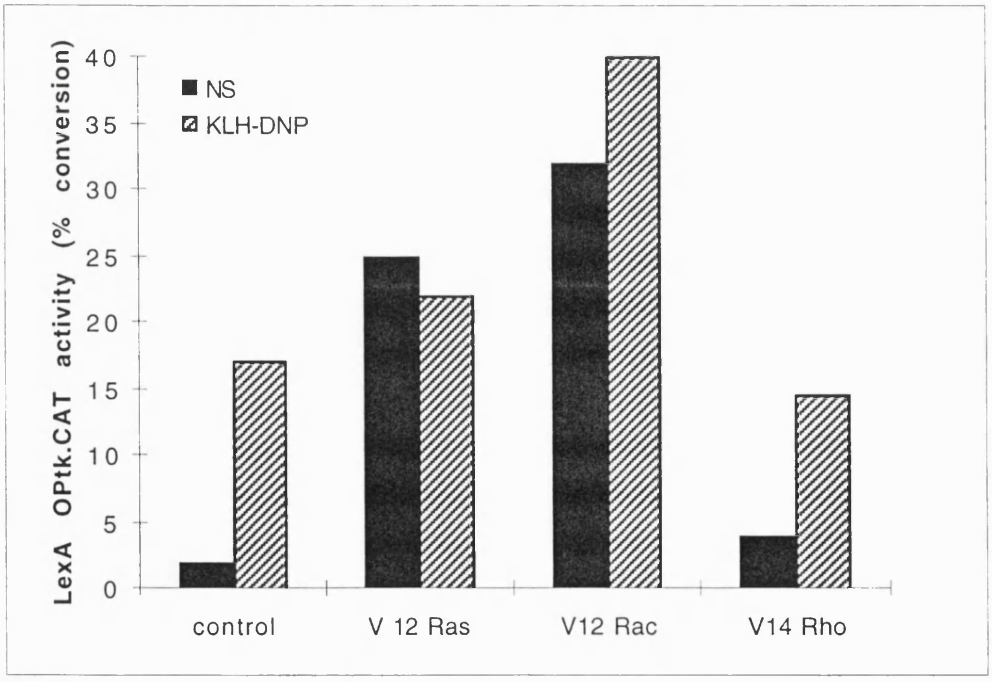


Figure 4.10. Activated mutants of Ras and Rac-1 but not RhoA induce Elk-1 transactivation in RBL2H3. $1x10^7$ RBL2H3 were electroporated with 12μg LexA OPtk.CAT reporter in combination with 6μg LexA Elk-1. Co-transfections were with pEF vector alone (control),

20μg pEF V12 Ras, 20μg pEF V12 Rac-1 or 20μg V14 RhoA. Cells were recovered for 6h then left unstimulated (NS) or treated with 250ng/ml KLH-DNP. Stimulation was for 14h before assay as described. Data are representative of 3 experiments.

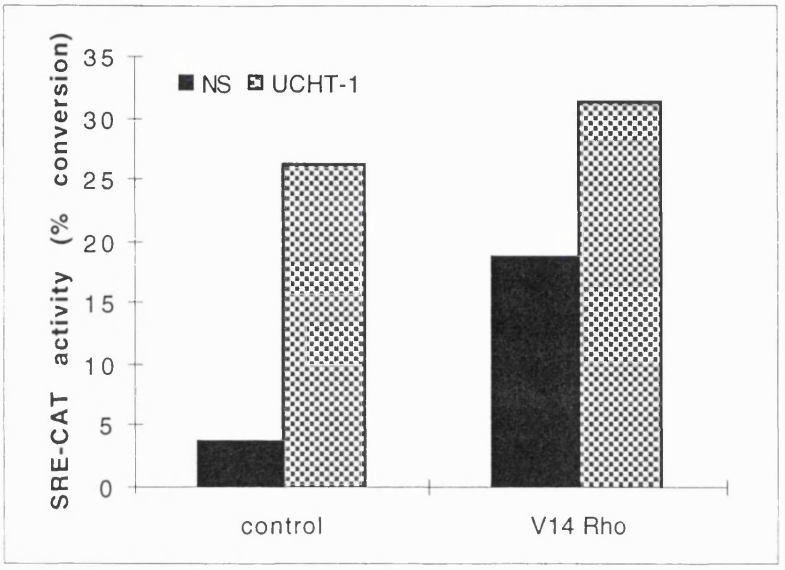


Figure 4.11. The activated V14 Rho mutant is able to induce activity of the Serum respoonse Element in Jurkat T cells. $1x10^7$ Jurkat JTAg cells were electroporated with 8μg of SRE-CAT reporter. Co-transfections were with pEF vector alone (control) or 20μg V14 RhoA. Cells were recovered for 6h then left unstimulated (NS) or treated with 2μg/ml UCHT-1. Stimulation was for 14h before assay as described.

Activated mutants of both Ras and Rac-1 stimulate Elk-1 transactivation in RBL2H3 in the absence of other stimuli. In contrast, V14 RhoA does not activate Elk-1. These data suggest that Ras and Rac-1may initiate signalling pathways which activate Elk-1 in RBL2H3 but that RhoA is unlikely to do so. Evidence that activated GTPase mutants can drive Elk-1 activity does not show that signalling by those GTPases is the mechanism by which a given receptor may accomplish this. Therefore further experiments were directed towards establishing whether FcεR1 activation of Elk-1 was dependent on the activity of Ras or Rac-1.

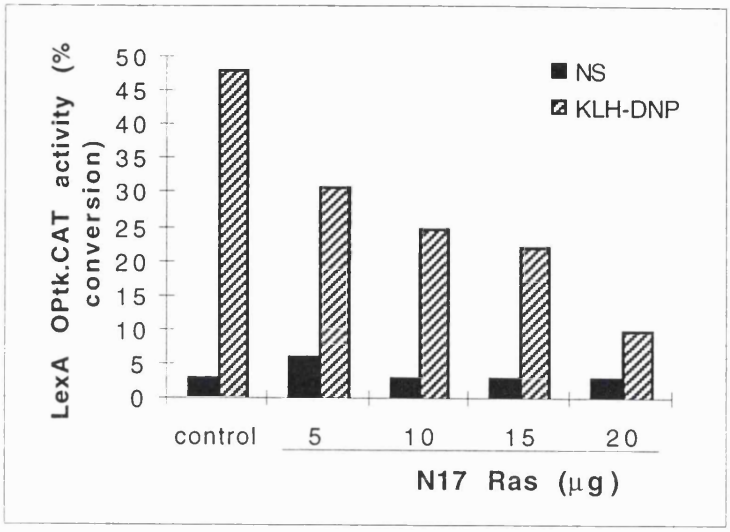
4.8 Dominant inhibitory mutants of Ras and Raf-1 but not Rac-1 inhibit FcεR1 mediated induction of Elk-1 transactivation.

The data presented so far establish that both V12 Ras and V12 Rac-1 can activate Elk-1 in RBL2H3. Dominant inhibitory mutants of Ras and Rac-1 were used to examine whether activity of one or both of these GTPases is required for FcεR1 regulation of Elk-1. The N17 mutants of Ras and Rac-1 act to sequester exchange factors away from endogenous pools of the GTPase and and hence maintain Ras or Rac in their GDP-bound, inactive state. The dominant negative (DN) Raf-1 mutant has the C-terminal kinase domain truncated; this mutant binds to activated Ras.GTP and

prevents its interaction with effector proteins. Hence, DN Raf-1 prevents interactions between Ras.GTP and endogenous Raf-1 and between Ras.GTP and other effector molecules. Therefore the effect of overexpression of DN Raf-1 would be expected to mimic N17 Ras in blocking all Ras mediated signalling pathways.

The data in Figure 4.12 show the effect of N17 Ras (a) or N17 Rac-1 (b) cotransfection on FcεR1 induction of Elk-1 transactivation in RBL2H3. N17 Ras inhibits FcεR1 induction of Elk-1 activity in a manner dose dependent on the amount of cotransfected N17 Ras plasmid. In contrast, cotransfection of N17 Rac-1 did not affect the ability of the FcεR1 to induce Elk-1 transactivation. A functional control for N17 Rac-1 efficacy was performed in that N17 Rac-1 cotransfection profoundly inhibited the FcεR1 induction of a reporter gene driven by the Nuclear Factor of Activated T cells (NFAT). Since Ras and Rac-1 regulation of NFAT are the major focus of Chapters 5 and 6 of this thesis, the significance of this control will not be discussed further here. The effect of the DN Raf-1 mutant on FcεR1 induction of Elk-1 was assayed and is shown in Figure 4.12c. The data show that FcεR1 induction of Elk-1 transactivation is markedly inhibited by co-transfection of truncated DN Raf-1 mutant. As expected, the DN Raf-1 mimics the effect of N17 Ras.

Figure 4.12 a



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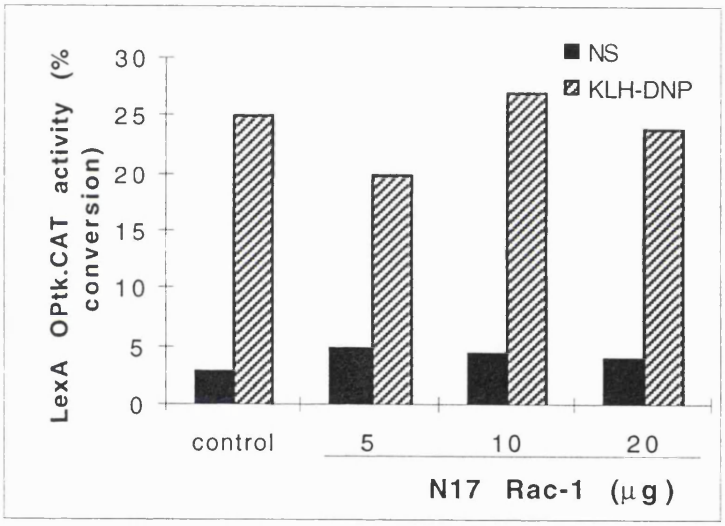

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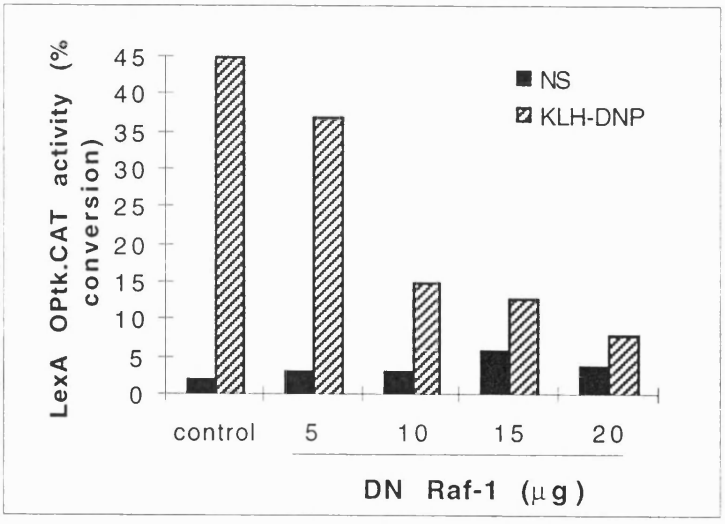


Figure 4.12. FcεR1 induction of Elk-1 transactivation is dependent on activity of Ras but not Rac-1. $1x10^7$ RBL2H3 were electroporated with 12μg LexA OPtk.CAT reporter in combination with 6μg LexA Elk-1. Co-transfections were with pEF vector alone (control), or the indicated microgram amounts of pRSV N17Ras, pEF N17 Rac-1 or pEF DN Raf-1 Cells were recovered for 6h then left unstimulated (NS) or treated with 250ng/ml KLH-DNP. Stimulation was for 14h before assay as described. Data are representative of 3 experiments.

These data suggest that FcεR1 induction of Elk-1 transactivation is dependent on Ras activity but not on Rac-1. While a pathway for Rac-1 activation of Elk-1 does exist in RBL2H3, as evidenced by the stimulatory effect of V12 Rac-1, this mechanism is not relevant for FcεR1 regulation of Elk-1 transactivation. Moreover, the data also show that of the possible Ras effector molecules which are known to transduce the immediate signal distal to Ras.GTP, it is highly unlikely that Rac-1 is involved in FcεR1 induction of Elk-1 in this fashion. In subsequent experiments, the role of the 'classical' Ras/Raf-1/MEK/ERK cascade in transduction of the FcεR1 signal to Elk-1 was investigated. Two reagents were used to assess the importance of the Raf-1/MEK/ERK cascade in FcεR1 regulation of Elk-1 and are shown schematically in Figure 4.13.

Ras.GTP induces membrane targeting and activation of Raf-1, which acts subsequently on the ERK MAP kinases via the ERK activating kinase MEK. The Raf-1 CAAX mutant comprises Raf-1 fused to a CAAX box motif which constitutively membrane localises Raf-1. Membrane localisation of Raf-1 is one strategy which has been shown to activate the Raf-1/MEK/ERK cascade in the absence of other stimuli. Used in cotransfection, this mutant would therefore be expected to activate a Raf-1/MEK/ERK dependent event in the absence of other stimuli (Leevers *et al.*, 1994). The other reagent shown in Figure 4.13 is the MEK inhibitor PD 098059. PD 098059 has been shown to be highly specific for the ERK activating kinase MEK. It binds MEK and inhibits its activation by c-Raf-1 *in vitro* with an IC 50 level of 4μM in KB cells (Alessi *et al.*, 1995). A large number of MEK-type kinases have been shown not to be targets for PD 098059, these include other MEK isoforms (MEK2) and homologues such as SEK1. Use of the MEK inhibitor PD 098059 should therefore remove the ability of a receptor to use the Raf-1/MEK/ERK pathway and establish if a downstream event is dependent on the activity of this cascade.

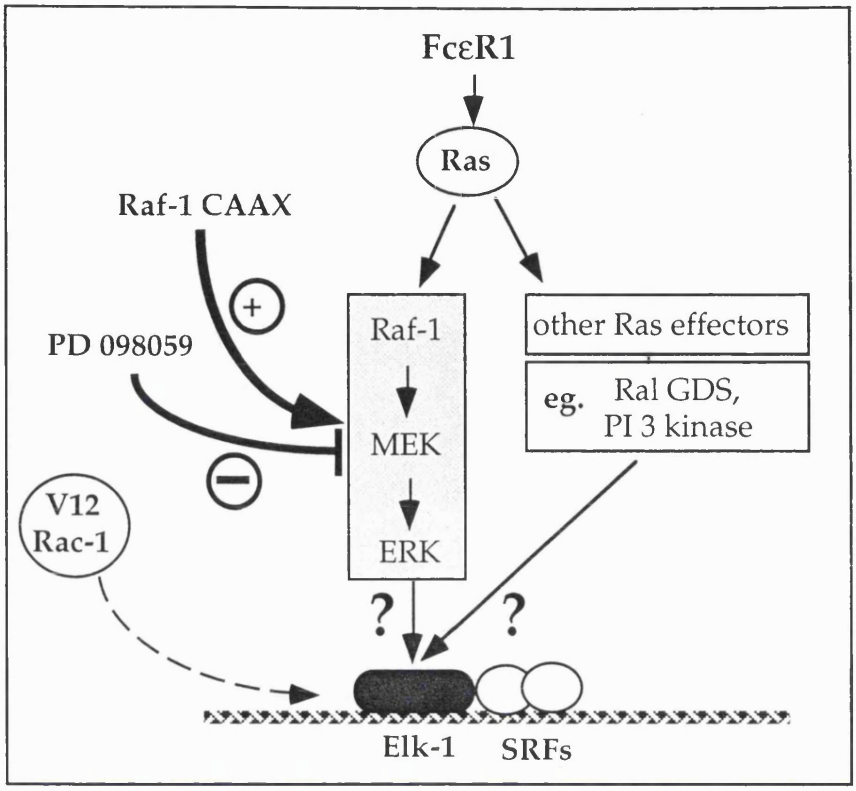


Figure 4.13. Elk-1 regulation in RBL2H3. Both V12 Ras and V12 Rac-1 stimulate Elk-1 transactivation in RBL2H3, although the FcεR1 regulation of Elk-1 is Rac-1 independent. Ras signals to Elk-1 may be transduced via the 'classical' Raf-1/MEK/ERK pathway or via one of the other characterised effectors of Ras. In other systems these have been characterised as including PI 3-kinase and a guanine nucleotide dissociation stimulator (GDS) protein for the Ral GTPase. The Raf-1 CAAX mutant acts to drive the Raf-1/MEK/ERK pathway in the absence of other stimuli. The small molecule inhibitor of MEK, PD 098059, will prevent FcεR1 use of the MEK/ERK cassette to regulate downstream targets.

4.9 FcεR1 regulation of Elk-1 is dependent on a functional Raf-1/MEK/ERK signalling cassette.

The aim of the experiments shown below was to ask if the FcεR1 regulation of Elk-1 was dependent on a functional Ras/Raf-1/MEK/ERK cascade. Initially an activated Raf-1 CAAX mutant was used to establish if stimulating activity of this cascade at a level below Ras itself could drive Elk-1 transactivation in RBL2H3. The data in Figure 4.14 show that co-transfection of the Raf-1 CAAX mutant strongly activates Elk-1 dependent reporter gene activity in the absence of other stimulation. This potentiation is strongly reminiscent of that observed with co-transfection of the V12 Ras mutant. These data suggest that activation of the Raf1/MEK/ERK pathway can mimic the effect of the FcεR1 on Elk-1 transactivation.

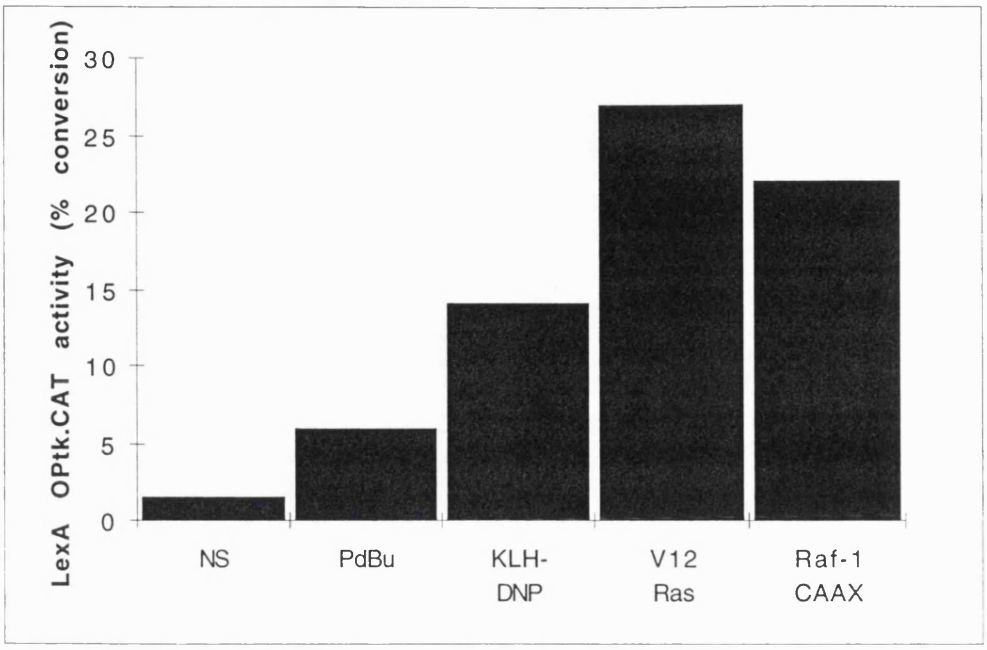


Figure 4.14. An active, membrane targetted form of Raf-1 can drive Elk-1 transactivation in RBL2H3. $1x10^7$ RBL2H3 were electroporated with 12μg LexA OPtk.CAT reporter in combination with 6μg LexA Elk-1. Co-transfections were with pEF vector alone (NS, PdBu, KLH-DNP), or 20μg of pef V12 Ras or pEF Raf-1 CAAX as indicated. Cells were recovered for 6h then left unstimulated (NS) or treated with 50ng/ml PdBu or 250ng/ml KLH-DNP. Stimulation was for 14h before assay as described. Data are representative of >3 experiments.

The role of the MEK/ERK cascade in FcεR1 stimulation of Elk-1 activity was explored directly using the MEK inhibitor PD 098059. The specificity of this inhibitor has been established extensively elsewhere. In initial experiments however, the effectiveness of PD 098059 as a MEK inhibitor in RBL2H3 was verified. The phosphorylation of ERK by MEK results in decreased electrophoretic mobility of ERK in SDS-PAGE. The data in Figure 4.15 show that both PdBu and FcεR1 crosslinking cause ERK phosphorylation and mobility shift in RBL2H3. Moreover, this effect is inhibited by PD 098059 in a dose dependent manner. At 25μM PD 098059, both PdBu-induced and KLH-DNP induced ERK shifts are returned to levels similar to that in control cells. These data establish the efficacy of PD 098059 as an inhibitor of ERK activation by MEK in RBL2H3.

The stimulatory effects of V12 Ras and Raf-1 CAAX mutants on Elk-1 transactivation are markedly inhibited by PD 098059 (Figure 4.16). These data indicate that the activity of these mutants can be attributed to their regulation of the MEK/ERK cascade. It seems highly likely that this is indeed the mechanism by which the FcεR1 regulates Elk-1.

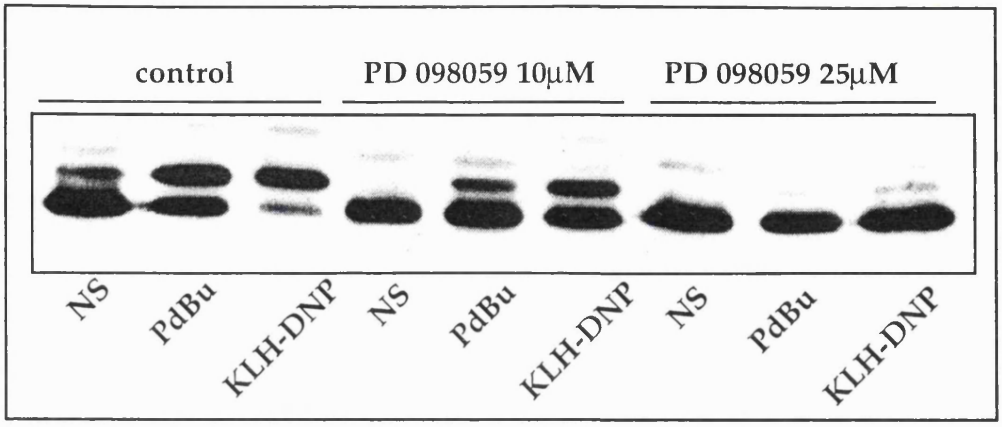


Figure 4.15. The MEK inhibitor PD098059 blocks FcεR1 and PdBu induced ERK hyperphosphorylation in RBL2H3. 10^6 RBL2H3 per point were incubated for 30 min with DMSO (control) or the indicated concentration of PD 098059. Stimulations were for 30 min with either 50ng/ml PdBu or 500ng/ml KLH-DNP. Cells were lysed and proteins were resolved by 15% SDS-PAGE. Western analysis for ERK was carried out using 1μg/ml anti pan-ERK.

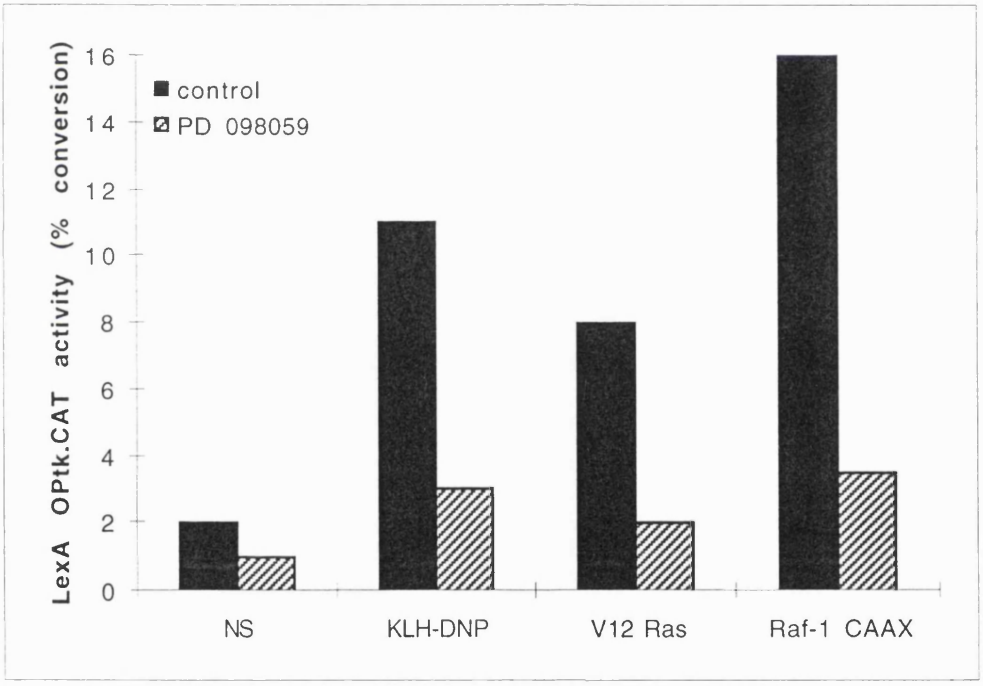
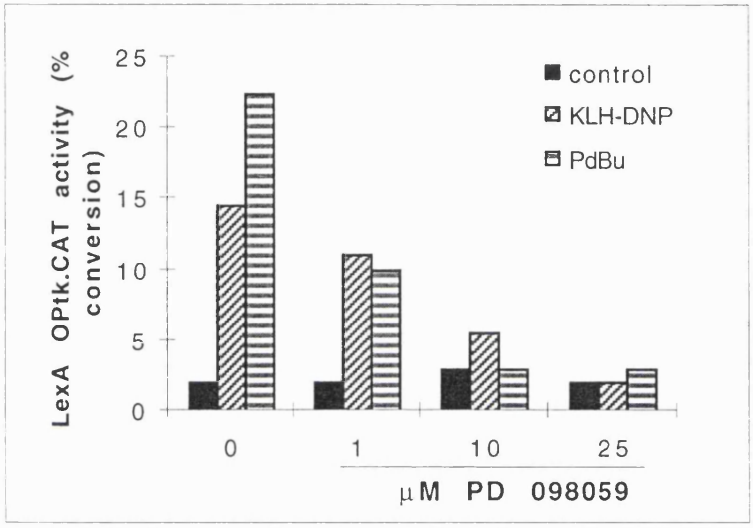


Figure 4.16. V12 Ras and Raf-1 CAAX effects on Elk-1 transactivation in RBL2H3 are mediated via the MEK/ERK pathway. $1x10^7$ RBL2H3 were electroporated with 12μg LexA OPtk.CAT reporter in combination with 6μg LexA Elk-1. Co-transfections were with pEF vector alone (NS, PdBu, KLH-DNP), or 20μg of pef V12 Ras or pEF Raf-1 CAAX as indicated. Cells were recovered for 6h then left unstimulated (NS) or treated with 50ng/ml PdBu or 250ng/ml KLH-DNP in the absence (control) or presence of 20μM PD 098059.. Stimulation was for 14h before assay as described. Data are representative of >3 experiments.

Accordingly, the effect of PD 098059 on Elk-1 transactivation in mast cells was examined. The data in Figure 4.17a show that PD 098059 inhibits the PdBu and FcεR1 induction of Elk-1 transactivation in a dose-dependent manner. In terms of % inhibition (Figure 4.17b) PB 098059 is able to inhibit PdBu and FcεR1 induction of Elk-1 by 80-85%. PdBu activation of Elk-1 transcriptional activity is apparently more sensitive to PD 098059. The IC 50 for PD 098059 inhibition of the PdBu stimulation is 1μM, whilst that for FcεR1 stimulation is approximately five-fold higher.

a



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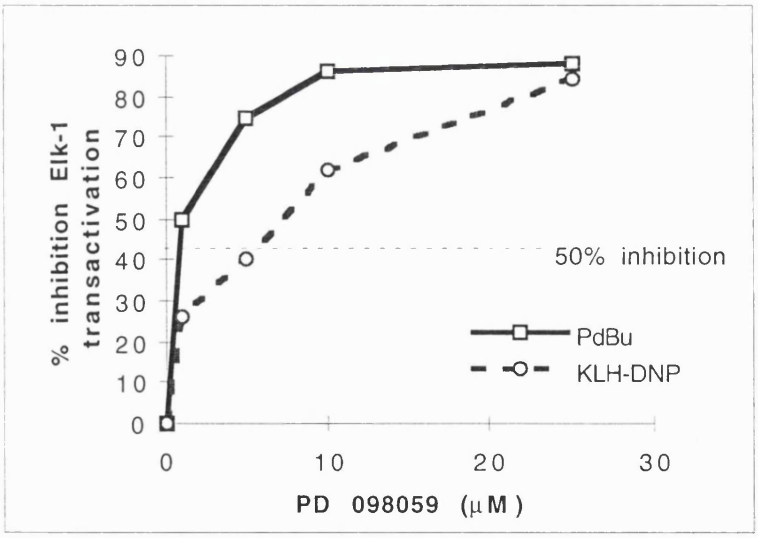


Figure 4.17. FcεR1 regulation of Elk-1 transactivation is dependent on the MEK-ERK pathway. $1x10^7$ RBL2H3 were electroporated with 12μg LexA OPtk.CAT reporter in combination with 6μg LexA Elk-1. Cells were recovered for 6h then left unstimulated (NS) or treated with 50ng/ml PdBu or 250ng/ml KLH-DNP in the absence (control) or presence of PD 098059 at the indicated concentrations. Stimulation was for 14h before assay as described. Data are representative of 3 experiments.

These data suggest that the transcription factor Elk-1 is a target for the Ras/Raf-1/MEK/ERK signalling pathway in RBL2H3. Thus Ras is placed upstream of a transcriptional regulator whose activity is induced in response to FcεR1 stimulation. By analogy with other systems, Ras is likely to have multiple effector pathways in RBL2H3. The data presented here show that the Raf-1/MEK/ERK cascade is the relevant effector pathway for Ras regulation of Elk-1 in response to FcεR1 stimulation of RBL2H3.

4.11 DISCUSSION.

FcεR1 regulation of gene transcription is an important facet of mast cell activation. The data presented in this Chapter have identified that the transcription factor Elk-1 is a target of signals initiated by antigenic crosslinking of the FcεR1. The generation of GTP loaded and hence active Ras is a critical step in the FcεR1 regulation of Elk-1. As summarised in Figure 4.18, the Ras.GTP regulation of Elk-1 provides a downstream target for the Grb2/Sos complexes characterised in Chapter 3. This model shows that activity of Sos recruited to the membrane by p33/Grb2 causes Ras to accumulate in the GTP loaded form. Amongst the multiple effector pathways which are likely to be activated by Ras.GTP is the 'classical' Raf-1/MEK/ERK cascade. The data presented in this Chapter have positively identified that the function of this signalling cascade is absolutely required for FcεR1 regulation of Elk-1. In FcεR1 regulation of RBL2H3, Elk-1 is a substrate for the ERK kinase and that phosphorylation of Elk-1 by ERK results in Elk-1 stimulation of gene transcription.

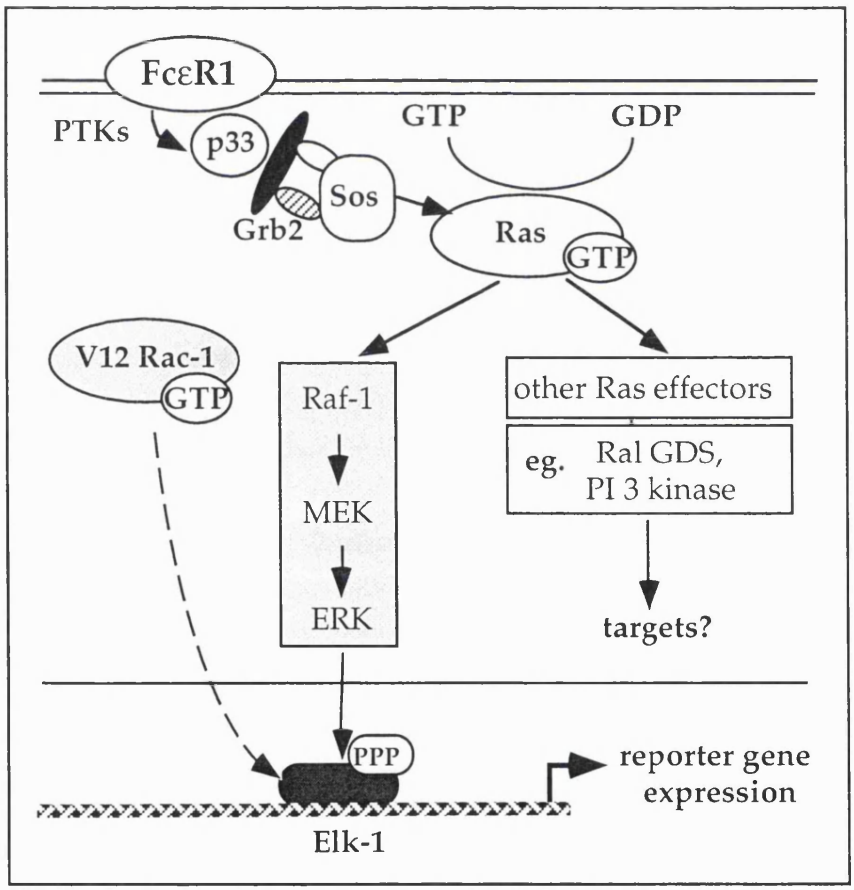


Figure 4.18. Elk-1 is a target for the Ras.GTP/Raf-1/MEK/ERK cascade in RBL2H3. The FcεR1 regulation of Elk-1 requires the activity of the Raf-1/MEK/ERK cascade. Ras GTP loading, promoted by the Sos Guanine Nucleotide Exchange Factor, is necessary and sufficient for Elk-1 transcriptional activity. A pathway for Rac.GTP activation of Elk-1 exists in RBL2H3, but is not the mechanism by which the FcεR1 controls Elk-1 activity.

4.12 Ras but not Rac-1 is required for FcεR1 activation of Elk-1 in RBL2H3.

The data generated using activated (V12) mutants of Ras and Rac-1 GTPases suggests that both mutants can drive Elk-1 transactivation in RBL2H3. However, when dominant inhibitory mutants of Ras and Rac-1 are used, only the blockade of Ras signals affects FcεR1 induction of Elk-1. Thus the V12 Rac-1 regulation of Elk-1 is not the relevant pathway for FcεR1 signalling. These data illustrate an important consideration of the use of activated GTPase mutants. Such mutants provide information on how an event can be controlled but not upon whether the process in question absolutely requires the activity of the GTPase. It is interesting to consider how Rac-1.GTP could control Elk-1. There is data from other cell systems that Rac-1 dependent signals can activate non-ERK MAP kinase family members and that these in turn can act upon Elk-1. It is possible that the strong Rac-1 signal delivered by overexpressed Rac-1.GTP can drive the activation of Elk-1 by JNK or possibly the p38 kinase. It is becoming clear that all Ras family GTPases have multiple effector molecules, a number of putative Rac effectors have been identified (reviewed in (Reif and Cantrell, 1997)) including the Pak family of serine/threonine kinases and MLK2,3, the tyrosine kinase $p120^{Ack}$, POR1 (partner of Rac1), $p67^{phox}$, Mek kinase (Mekk)-1,4 or phosphatidylinositol 4-phosphate 5-kinase (PI4P 5-kinase). A consequence of V12 Rac over-expression is likely to be the simultaneous activation of numerous effector pathways; differing from the physiological situation where a receptor-derived signal will select for a limited number of effectors. Hence the V12 Rac activation of Elk-1 in RBL2H3 may reflect the activation of an effector pathway not normally induced by FcεR1 activation of endogenous Rac-1 protein.

4.13 Ras regulation of Elk-1 in RBL2H3 requires the function of the 'classical' Raf-1/MEK/ERK pathway.

Extensive studies have shown that transcriptional activation of Elk-1 is dependent on its C-terminal phosphorylation by members of the MAP kinase family such as Erk-1/2, the Jun (stress-activated) kinase JNK and the p38 kinase. Activation of the MAP kinases Erk1/2 is mediated by the Ras/Raf-1/MEK cascade. Despite the potential for Elk-1 to be targeted by multiple MAP kinases, the data herein show that the Ras effector pathway involved in FcεR1 regulation of Elk-1 absolutely requires the activity of the Erk-activating kinase MEK, acting downstream of Ras and its effector Raf-1. These data show that the Ras/Raf-1/MEK pathway has a dominant role in FcεR1 regulation of Elk-1, reflecting clear parallels between Elk-1 activation following antigen receptor ligation and growth factor stimulation of fibroblasts.

The regulation of Elk-1 by Ras is likely to be only one facet of the role of Ras in FcεR1 signalling. Ras activation of multiple effector pathways is recognised in other signalling systems (Marshall, 1996). However, no direct targets for Ras in the FcεR1 system have previously been identified. It seems likely that as more Ras dependent events are characterised, there will be multiple mechanisms distal to Ras that are used to regulate target molecules. Moreover, one of the characteristics of the complex effector networks which are apparently employed by GTPases is that the facility exists for multiple outcomes to a single input. In turn this means that the thresholds for functional events to occur can be very finely controlled. In a system such as FcεR1 signalling, where inappropriate target activation can cause pathology, such networks may be highly significant.

4.14. Functional significance of Elk-1 activation for the FcεR1.

A very limited number of gene targets for Elk-1 are currently known (Bassuk and Leiden, 1997). In hematopoietic cells, only the c-*fos* and c-*egr* proto-oncogene promoters are identified Elk-1 targets. ETS-family transcription factors as a whole have a large number of gene targets which encode significant imune response molecules. For example, the genes for the Class I MHC molecule, a variety of cytokine receptors and integrins such as ICAM-1 are regulated by ETS family members other than Elk-1.

The protein product of the c-*fos* proto-oncogene is a transcription factor and member of the AP-1 family of proteins. AP-1 proteins homo- and hetero-dimerise to form functional transactivating complexes which regulate numerous promoters (Karin, 1994; Karin and Hunter, 1995). In general, AP-1 dimers bind DNA in close conjunction with other transcription factors, this co-operation may be required for transcriptional induction by the binding partner of the AP-proteins. This type of interaction may have considerable functional significance for FcεR1 regulation of mast cell activation,

Members of the Nuclear Factor of Activated T cells (NFAT) family of transcription factors regulate a number of genes encoding significant immune proteins. These include cytokine genes such as IL-2, IL-4, IL-3 and the genes encoding the Fas- and CD40-ligand molecules (Rao *et al.*, 1997). Induction of IL-2 and IL-4 transcription in T cells has been extensively studied and requires activity of NFAT and a dimer of AP-1 binding proteins (Jain *et al.*, 1993; Rooney *et al.*, 1995). While there are multiple AP-1 proteins, it seems clear that the relevant dimer for NFAT/AP-1 complex formation in this system is formed of Fos and Jun. Therefore, in T cells at least, production of IL-4 is dependent on the presence of Fos protein. In this context it is reasonable to suggest that any FcεR1 regulation of Fos production could contribute to the induction of cytokine synthesis. Fos gene induction by the FcεR1 (Stephan *et*

al., 1997) has been described but Fos induction in RBL2H3 has not been explored in this thesis. This is in part due to the extremely low levels of induction which can be achieved by FcεR1 regulation of a reporter gene driven by the whole Fos promoter in RBL2H3.

The contribution of Elk-1 regulation to FcεR1 activation of mast cells can be summarised as follows. 1) the likely Elk-1 regulation of the Fos promoter can contribute to the AP-1 regulation of cytokine gene induction and 2) other as yet uncharacterised Elk-1 target genes may contribute to the wide complement of responses which together comprise mast cell activation. Figure 4.19 shows a putative mechanism for the contribution of Elk-1 to IL-4 induction.

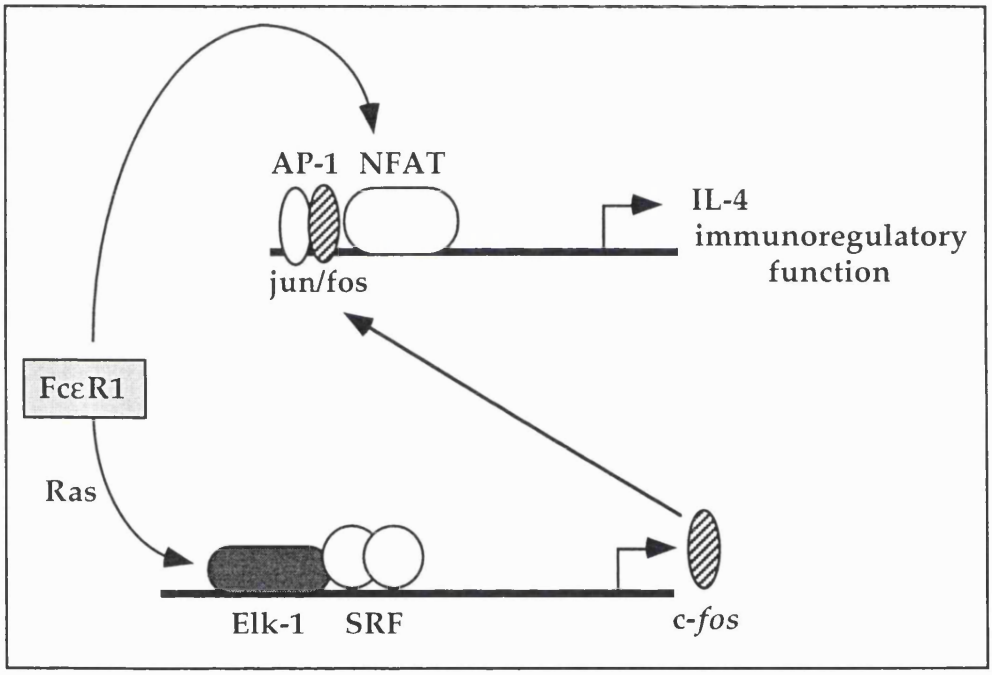


Figure 4.19. Elk-1 activation of the Fos gene may contribute to cytokine gene induction by the FcεR1. The FcεR1 regulates the Elk-1 transcription factor, one target of which is the c-*fos* gene. Fos protein contributes to the formation of a functional NFAT/AP-1 complex which is involved in the induction of immunoregulatory genes such as that for IL-4. NFAT: Nuclear Factor of Activated T cells, IL: Interleukin, SRF: Serum response Factor.

The data presented in this Chapter have explored the role of Ras family GTPases in the induction of Elk-1 transcriptional activity in mast cells. The Ras/Raf-1/MEK pathway links the FcεR1 to at least one regulatory factor for the induction of immediate early genes. These results lend a functional significance to the previous biochemical observations that regulation of the Sos exchange factor is a target of a major FcεR1 signalling pathway. Immediate early genes are not the only transcriptional targets of FcεR1 signals. FcεR1 stimulation of mast cells results in the induction of multiple genes for cytokines, chemokines and growth factors. The signalling pathways which control induction of genes of these classes have not been studied. The work to be presented in Chapters 5 and 6 examines the role of Ras family GTPases in the FcεR1 regulation of the Interleukin-4 promoter.

CHAPTER FIVE

Signals regulating IL-4 promoter elements in mast cells include targets for Ras family GTPases and calcium.

5.1 INTRODUCTION

In response to FcεR1 stimulation of mast cells, allergic mediators are released and *de novo* synthesis of various gene products is induced. The data presented in Chapter 4 have shown that FcεR1 regulation of the Ras GTPase is critical for induction of Elk-1 transcriptional activity and hence for the regulation of certain classes of immediate early gene promoters. Induction of cytokine gene transcription by the FcεR1 is an important facet of mast cell activation. Upon FcεR1 stimulation various cytokines are produced, including Interleukin (IL-) 4, IL-3, IL-6, the Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and Tumor necrosis factor (TNFα) (Jouvin *et al.*, 1995; Plaut *et al.*, 1989; Rao *et al.*, 1997). The data presented in this Chapter will detail initial experiments using a reporter gene assay to elucidate the nature of FcεR1 signals which regulate the IL-4 gene promoter region. The data show that a reporter gene driven by a minimal IL-4 promoter region is a target for Ras, Rac-1 and calcium signals in RBL2H3. By analogy with T cell antigen receptor regulation of the IL-2 and IL-4 promoter regions, NFAT/AP-1 transcription factor complexes were identified as likely targets for calcium/GTPase signals in the RBL2H3. Accordingly, reporter gene assays for activity of NFAT/AP-1 complexes were established.

5.1.1 Mast cell derived cytokines and chemokines are important for initiation and maintenance of an inflammatory response.

In response to FcεR1 ligation mast cells induce the expression of several classes of gene, including immediate early genes and those encoding multiple cytokines and chemokines. The induction of chemokine and cytokine gene transcription by the FcεR1 is required for the initiation and maintenance of an inflammatory response. Chemotactic gradients are generated which facilitate the infiltration of secondary leukocyte populations into a developing inflammatory site. Mast cell derived cytokines are then in a position to influence the differentiation and activation status of these infiltrating leukocytes (Mecheri and David, 1997).

Mast cells produce high levels of Interleukin-4 in response to FcεR1 ligation (Plaut *et al.*, 1989). The importance of this cytokine was described in Chapter 1. IL-4 will act to promote isotype switching of B cells to IgE, a feedback mechanism for

maintenance of IgE dependent mast cell activation. Moreover, IL-4 will influence the differentiation of helper CD4+ T lymphocytes which infiltrate the inflammatory site. In combination with absence or low levels of IFNγ, IL-4 promotes Th differentiation into a Th2 phenotype. Commitment to the Th2 phenotype has important consequences for the development of pathology. In the context of allergen challenge or helminth infection the Th2 phenotype contributes to the onset of an allergic reaction or a curative anti-parasite response respectively. IL-4 is clearly an important determinant of the outcome of immune responses initially triggered by antigenic crosslinking of the FcεR1 on mast cells. The FcεR1 signalling pathways which regulate the IL-4 promoter have not been examined. In order to study this aspect of mast cell activation, an assay for activity of the IL-4 promoter in RBL2H3 was developed.

5.1.2 Architecture of the IL-4 promoter.

The induction of gene expression is controlled by sequence-specific binding of regulatory factors to DNA upstream of the transcriptional start site delineated by the TATA box. The work of Chuvpilo *et al* (1993) identifies that a region from -12 to -270 of the murine IL-4 gene contains a strong transcriptional enhancer that is capable of driving transcription of a CAT reporter gene. DNAse I hypersensitivity assays showed that six sites in this 270bp region can bind to nuclear proteins. Four of these regions are purine rich boxes which share the motif GGAAA, a consensus site for the Nuclear Factor of Activated T cells (NFAT) transcription factor. These sites were designated Purine boxes A-D (PuB_{A-D}), where A is most proximal to the transcriptional start site. Purine boxes B, C and D also contain weak binding sites for Octamer/AP-1 transcription factors. The remaining two protein bound regions of the IL-4 -270 region were designated Box I and Box II, where Box I is closest to the TATA box. Box I and II are found to bind Nuclear Factor (NF) (Y) and High Mobility Group I (HMG 1) factors respectively. Interestingly, Electrophoretic Mobility Shift Assays (EMSA) show that HMG-1 protein can bind certain Purine boxes as well as the Box II region. When HMG-1 is overexpressed in cells bearing an IL-4 promoter reporter gene construct, activity of the promoter is supressed. These data suggest that HMG-1 can compete for NFAT binding sites and act as a negative regulator of the activity of the IL-4 promoter.

The architecture of the IL-4 promoter is shown schematically in Figure 5.1, with those factors shown to bind regions of the promoter by EMSA indicated.

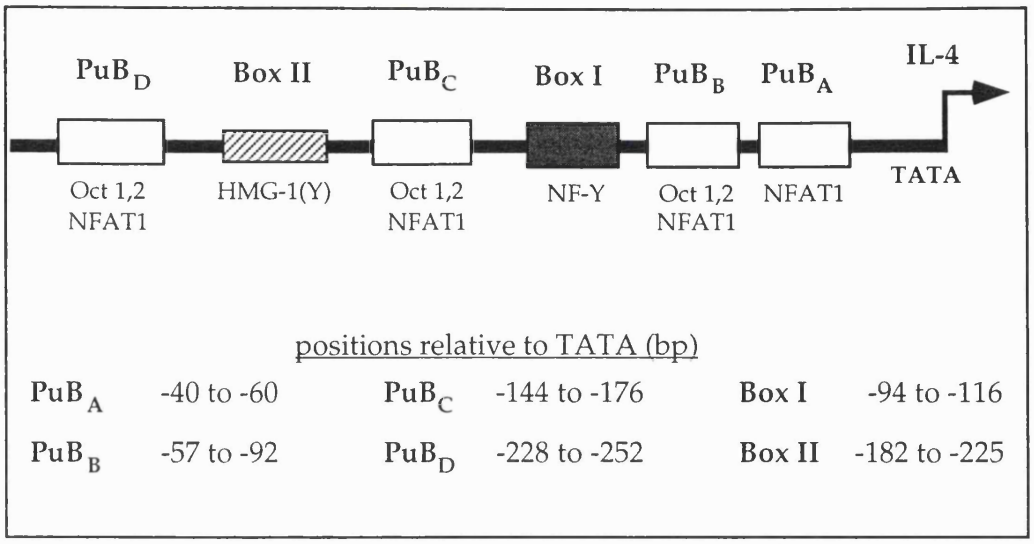


Figure 5.1. Architecture of the murine IL-4 promoter. Binding sites for regulatory factors demonstrated by EMSA are shown schematically. Their positions as delineated by DNAse I hypersensitvity assay are shown in base pairs (bp) from the TATA start site. PuB, purine box; NFAT, Nuclear Factor of Activated T cells; HMG, high mobility group; Oct, Octamer factor; NF, nuclear factor.

While T cells produce IL-4, it is the regulation of the IL-2 promoter/enhancer region which is best studied in this system. The promoters of the two genes show considerable similarity in the transcription factors which they bind. The most marked difference is in the lack of an NFκB site in the IL-4 promoter. The regulation of the IL-2 promoter by the T cell antigen receptor has been examined by a number of groups. The signals which have been identified to act upon elements binding to this region to date include 1) the activity of Protein kinase C , 2) signals derived from Ras family GTPases and 3) activation of the calcium dependent phosphatase calcineurin (CN). IL-2 gene induction is controlled by at least 8 regulatory factors binding the promoter/enhancer region (Jain *et al.*, 1995b; Rao, 1995; Serfling *et al.*, 1995).. Of these, the activity of a transcriptional complex composed of NFAT and AP-1 factors is absolutely required for IL-2 gene induction .

5.1.3 The NFAT/AP-1 complex regulates cytokine gene promoter activity.

NFAT proteins and their regulation will be described in more detail in the introduction to Chapter 6. NFAT binds co-operatively with a dimer of AP-1 family proteins to a composite site within cytokine gene promoter regions (Jain *et al.*, 1993; Rao, 1994). AP-1 dimers are ubiquitous, nuclear and newly synthesised in response to TCR ligation. The interaction with AP-1 is essential for NFAT transcriptional activity. Reporter genes constructed with point mutations in either the NFAT or AP-1

site are not inducible by pharmacological or antigenic stimuli in T cells. There is considerable data that the two components of the NFAT/AP-1 complex are differently regulated. The role of Ras signals in NFAT/AP-1 activation has been ascribed to the ability of Ras to stimulate AP-1 activity in T cells (Rayter *et al.*, 1992; Woodrow *et al.*, 1993). AP-1 dimers, typically composed of the Fos and Jun molecules, are resident within the nucleus. In contrast, NFAT protein is localised to the cytosol in resting cells and is translocated to the nucleus in a calcium dependent fashion following TCR ligation. The nuclear import of NFAT protein is regulated by the calcium-sensitive phosphatase Calciunerin (CN). For this reason, NFAT/AP-1 complexes have been the focus of a great deal of study: CN is the target of the macrolide immunosupressant drugs Cyclosporin A and FK506 (Rao *et al.*, 1997; Schreiber and Crabtree, 1992).

5.1.4 Aims.

FcεR1 regulation of the IL-4 gene is an important aspect of mast cell activation. Functionally, mast cell derived IL-4 contributes to the initiation and maintenance of an inflammatory response. The IL-4 promoter region contains numerous regulatory elements, some of which are also found in the IL-2 promoter, the latter having been more extensively studied. The aims of the work presented in this Chapter are:

1) To establish an assay for FcεR1 regulation of IL-4 promoter activity in the RBL2H3 cell line.

2) To examine the FcεR1 regulated signalling pathways which are involved in control of IL-4 promoter activity.

5.2 RESULTS

5.2.1 IL-4 (-270) CAT is a reporter for IL-4 promoter activity in RBL2H3.

A region 270 base pairs upstream of the TATA box of the murine IL-4 promoter contains binding sites for a large number of regulatory transcription factors (shown schematically in Figure 5.1). A construct comprising this 270bp region cloned upstream of a CAT reporter gene (Chuvpilo *et al.*, 1993) was obtained from Professor Edgar Serfling (University of Wurzberg). Initial experiments optimised transient transfection conditions for this reporter in RBL2H3. In these assays DNA concentration and time of exposure to the calcium ionophore Ionomycin. The data in Figure 5.2 show that in response to Ionomycin stimulation of RBL2H3, the optimal fold increase in IL-4 (-270) CAT activity is observed after 16h stimulation of cells transfected with 25μg reporter DNA. The inducibility of CAT expression driven by this promoter region is not high (3-4) fold. There is significant basal CAT gene activity. Moreover, at longer time points (>24h stimulation), the differential between CAT activity in non-stimulated and stimulated cells decreases sharply. Hence, it was necessary to carefully control the transfection conditions and time of stimulation in order to work with this reporter construct.

The data in Figure 5.3 show that elevation of intracellular free calcium by application of the ionophore Ionomycin induces activity of the IL-4 (-270) CAT reporter gene construct in RBL2H3. The phorbol ester PdBu does not induce IL-4 (-270) CAT activity in RBL2H3. Moreover, PdBu addition does not potentiate the effect of ionomycin on induction of IL-4 (-270) CAT activity. These data do not resolve the issue of whether PKC signals target the IL-4 (-270) region, the maximal stimuli observed with ionomycin are likely to mask any additional requirement for another signalling pathway. In order to examine the FcεR1 signalling pathways which are important for IL-4 promoter induction in RBL2H3, it was necessary to demonstrate induction of IL-4 (-270) CAT activity by antigenic crosslinking of the FcεR1.

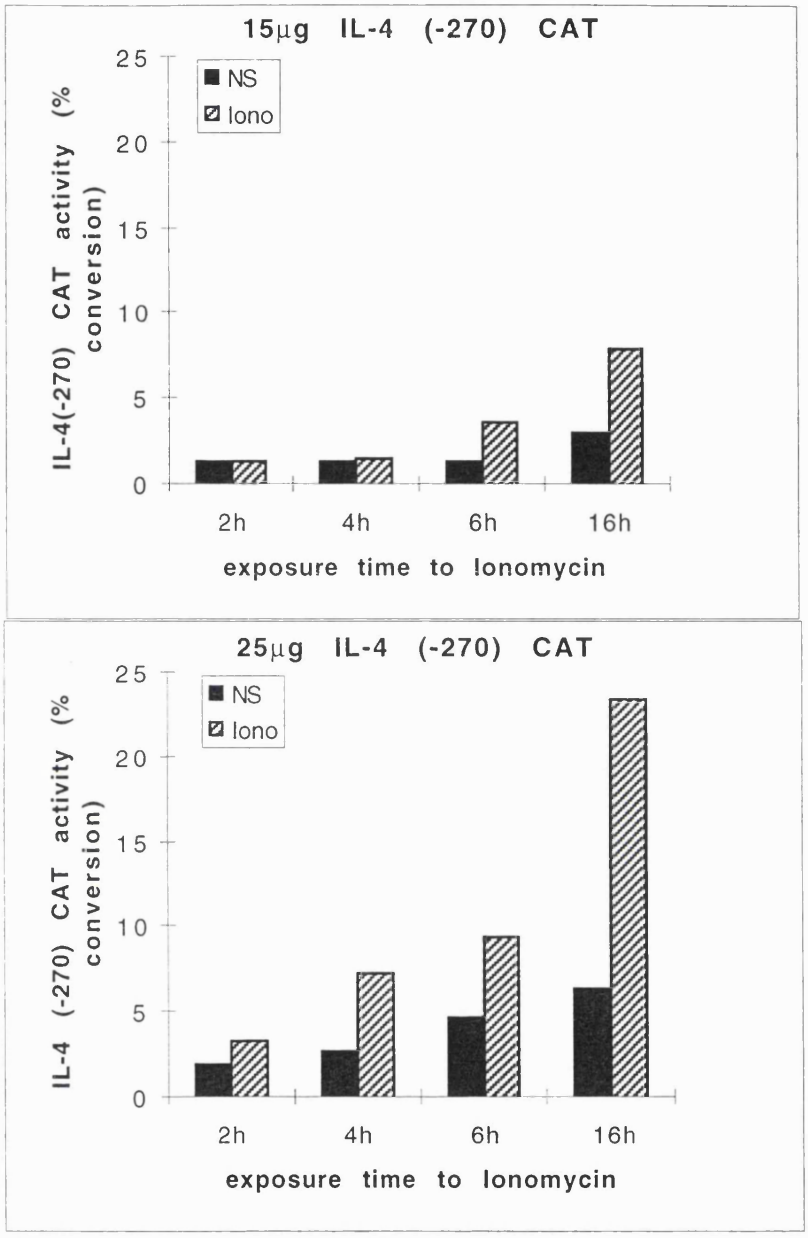


Figure 5.2. Optimal transfection conditions for the use of the IL-4 (-270) CAT reporter gene in RBL2H3. 10^7 RBL2H3 per point were transfected with the indicated amounts of reporter gene. Cells were pooled and aliquotted before stimulation for the indicated time with 500ng/ml Ionomycin. At timepoints cells were washed into ice cold PBS. All samples were assayed for CAT activity as described.

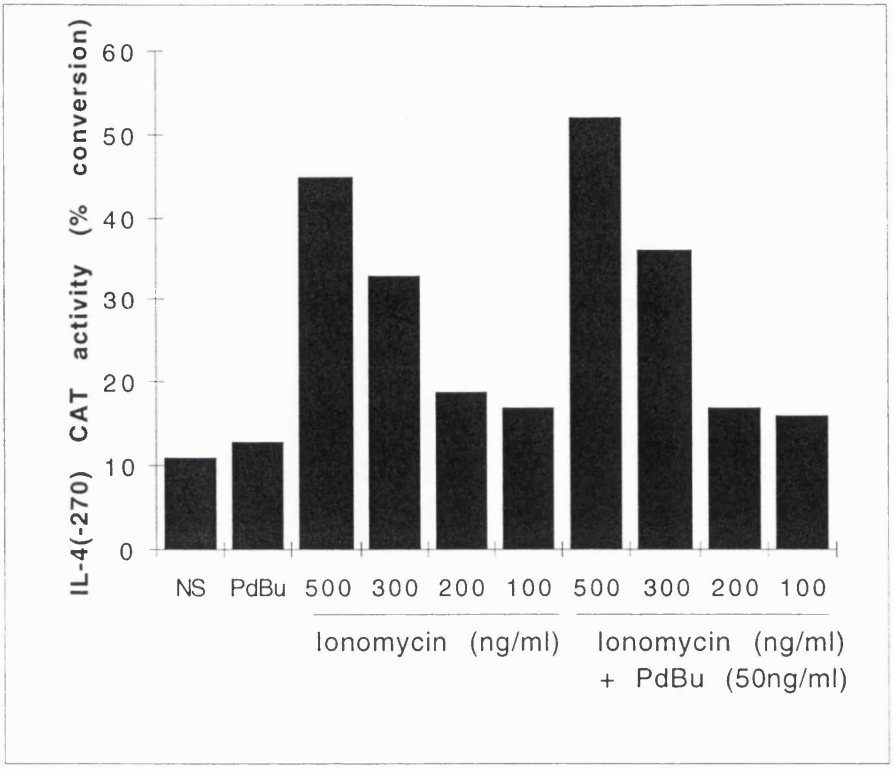


Figure 5.3. Ionomycin, but not Phorbol ester stimulation induces IL-4 (-270) CAT activity in RBL2H3. 10^7 RBL2H3 per point were transfected with 25μg reporter and receovered for 1h before stimulation with the indicated concentrations of ionomycin alone or in combination with 50ng/ml PdBu. After 16h stimulation cells were harvested and CAT activity was assayed. Data are representative of three experiments.

The data in Figure 5.4 show that IL-4 (-270) CAT activity is induced by antigenic stimulation of the FcεR1, in a manner dose-dependent on the amount of crosslinking antigen applied. The maximal fold increases over basal activation observed in response to IgE/antigen stimulation were reproducibly 3-4 fold with high doses of crosslinking antigen. Typical inductions in response to ionomycin were similar to those seen with antigen. These inductions do not represent a large window for the study of signals regulating the IL-4 (-270) region using either a cotransfection approach similar to that taken in Chapter 4 to examine Elk-1 regulation or chemical inhibitors of signalling proteins. Nevertheless, some analysis of the signalling pathways regulating this region was undertaken, prior to the identification of key regulatory elements within the promoter which were studied in more detail.

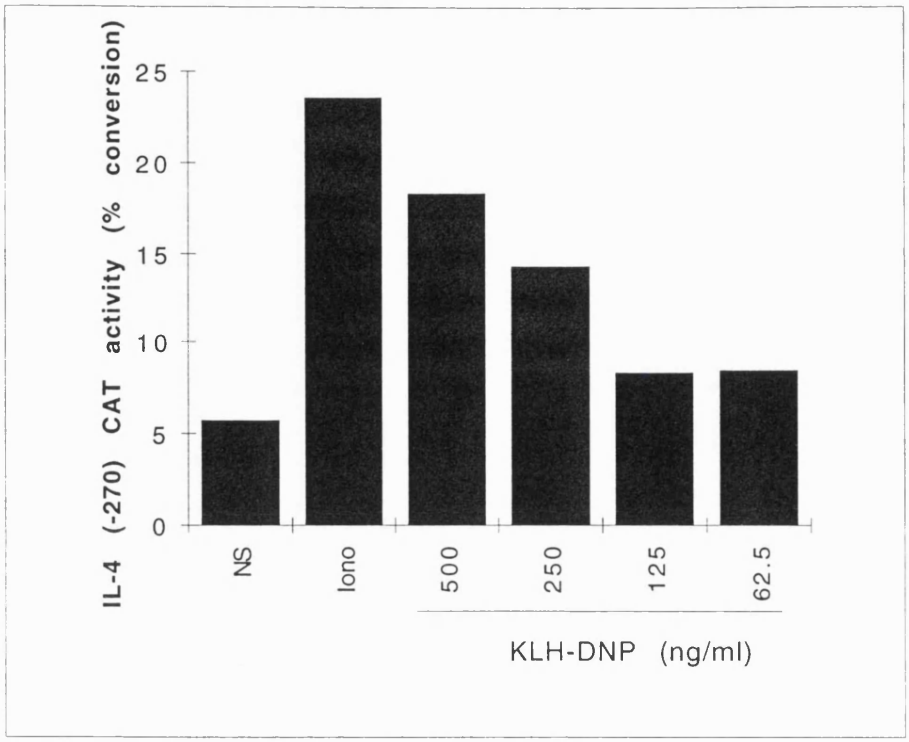


Figure 5.4. Antigenic crosslinking of the FcεR1 induces IL-4 (-270) CAT activity in RBL2H3. 10^7 RBL2H3 per point were transfected with 25μg reporter and recovered for 1h before either priming with IgE at 1μg/ml for 1h and stimulation with the indicated concentrations of KLH-DNP or stimulation with 500ng/ml ionomycin. After 16h stimulation cells were harvested and CAT activity was assayed. Data are representative of three experiments.

5.2.2 Signals transduced by Ras family GTPases are important for FcεR1 induction of IL-4 (-270) CAT activity.

The data in Figure 5.3 show that calcium signals are capable of strongly inducing activity of the IL-4 promoter in RBL2H3. Other candidate signals of interest which could link the FcεR1 and the IL-4 promoter include the activity of Ras family GTPases. In the T cell antigen receptor system, the regulation of the IL-2 promoter has been extensively studied. In this system, Ras and calcium signals synergistically induce activity of the IL-2 promoter. This effect is known to be based upon Ras and calcium activation of the NFAT/AP-1 transcription factor complex. The regulation of this complex in the context of the IL-4 promoter will be returned to in detail later. However, it is clear that Ras signals are important regulators of cytokine gene promoters in antigen receptor regulated T cells. Therefore the effect of activated and dominant inhibitory Ras upon FcεR1 induction of IL-4 (-270) CAT activity was assessed.

The data in Figure 5.5 show the effect of dominant inhibitory (N17) and activated (V12) Ras mutant cotransfection on the FcεR1 induction of IL-4 (-270) CAT activity in RBL2H3. In the presence of dominant inhibitory N17 Ras, the ability of either ionomycin or IgE/antigen to induce IL-4 (-270) CAT activity is severely reduced. In contrast, cotranfection of the activated V12 Ras mutant induces IL-4 (-270) CAT activity in the absence of other stimulation. The V12 Ras induction of IL-4 (-270) CAT activity is equivalent to that seen with IgE/antigen stimulation. These data suggest that Ras mediated signals may well be important for FcεR1 regulation of the IL-4 promoter enhancer region in RBL2H3.

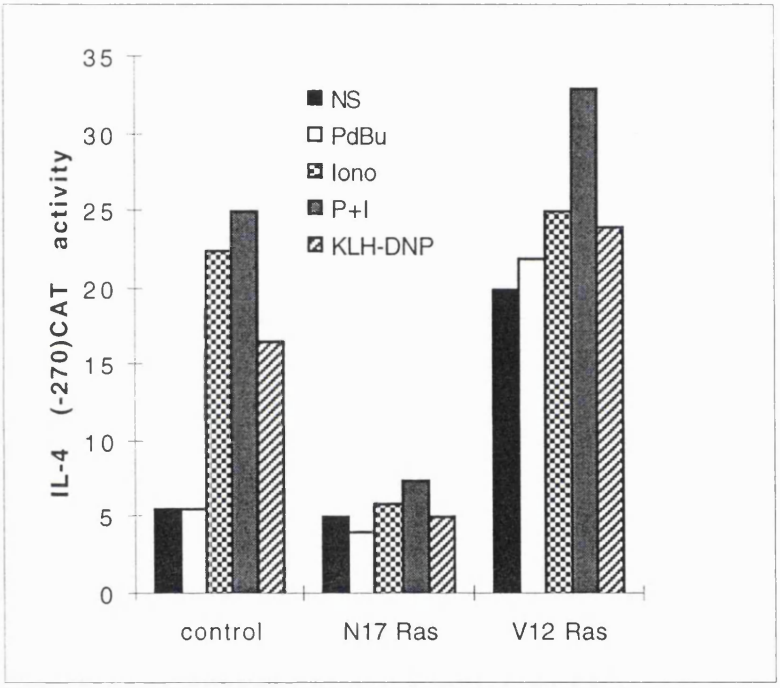


Figure 5.5. Co-transfection of mutants of the Ras GTPase affects FcεR 1 induction of IL-4 (-270) CAT activity in RBL2H3. 10^7 RBL2H3 per point were transfected with 20μg reporter alone or in combination with an equivalent amount of the indicated mutant Ras construct. Cells were recovered for 6h before stimulation as indicated. Concentrations of stimuli used were as follows; Ionomycin, 500ng/ml; PdBu, 50ng/ml; KLH-DNP, 500ng/ml. After 16h stimulation cells were harvested and CAT activity was assayed. Data are representative of three experiments.

As described in Chapter 4, any description of a possible role for Ras requires an analysis of the possible effector pathway used to transduce the Ras signal. The best characterised Ras effector pathway is the 'classical' Raf-1/MEK/ERK signalling cascade. In the RBL2H3 this pathway is the Ras effector cascade relevant to the FcεR1 induction of Elk-1 transcriptional activity. Hence, the effect of activation or inhibition of this signalling pathway on FcεR1 induction of IL-4 (-270) CAT activity

was assessed. The data in Figure 5.6 show the results of such an experiment. The MEK inhibitor PD 098059 was used as described in Chapter 4 to examine the MEK dependence of IL-4 (-270) CAT induction by the FcεR1. In control cells, FcεR1 stimulation by IgE/antigen resulted in a 3 fold induction of CAT reporter activity. In cell treated with PD 098059, this induction was severely reduced. These data would seem to suggest that the Raf-1/MEK/ERK cascade is important for FcεR1 induction of IL-4 (-270) CAT. Activation of this pathway using the Raf-1 CAAX mutant, which strongly induces Raf-1/MEK dependent Elk-1 activity, did not cause an induction of IL-4 (-270) CAT activity above basal levels.

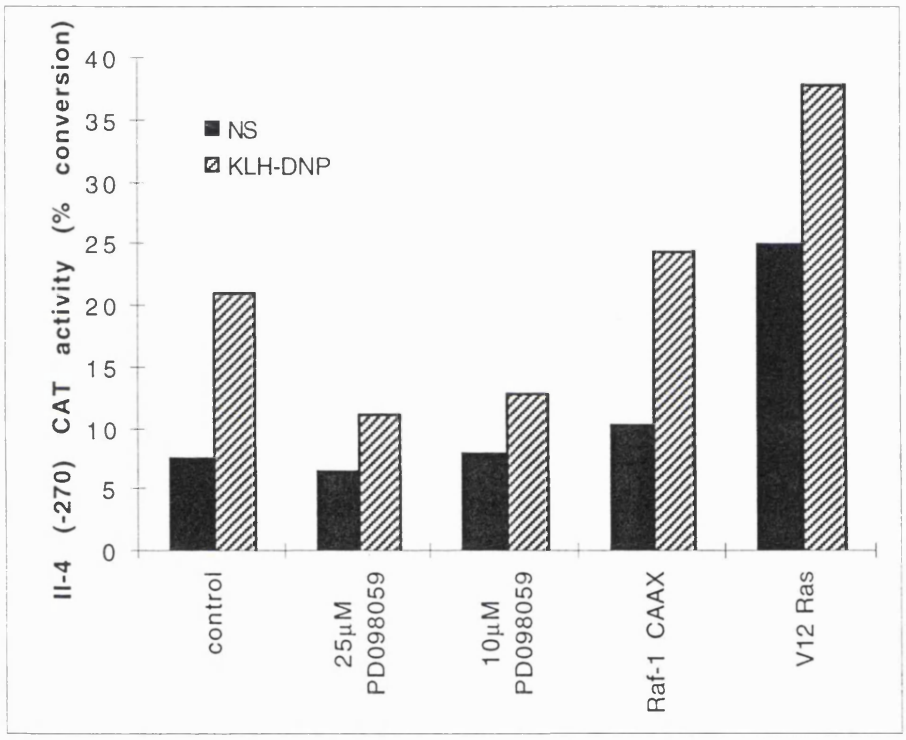


Figure 5.6. The Raf-1/MEK effector pathway is involved in FcεR1 induction of IL-4 (-270) CAT activity in RBL2H3. 10^7 RBL2H3 per point were transfected with 20μg reporter alone or in combination with an equivalent amount of the pEF Raf-1 CAAX or pEF V12 Ras. Cells were recovered for 6h before IgE priming and stimulation as indicated with 500ng/ml KLH-DNP. Where indicated cells were pre-treated with the MEK inhibitor PD 098059. After 16h stimulation cells were harvested and CAT activity was assayed. Data are representative of three experiments.

Since Rac-1 is 1) a known regulator of elements of the IL-2 promoter and 2) a possible Ras effector molecule other than Raf-1, the effect of activated and dominant inhibitory Rac-1 mutants on FcεR1 induction of the IL-4 (-270) CAT construct was assessed. The data in Figure 5.7 show that in the presence of the dominant inhibitory N17 Rac-1, the ability of either phorbol ester/ionomycin or IgE/antigen stimulation to induce IL-4 (-270) CAT activity was reduced to basal levels. The activated V12 Rac-1

mutant was able to induce IL-4 (-270) CAT reporter gene activity in the absence of other stimulation, but no additive effect of this mutant GTPase with FcεR1 stimulation was observed. These data suggest that in addition to Ras mediated signals, Rac-1 targets regulatory elements of the IL-4 promoter.

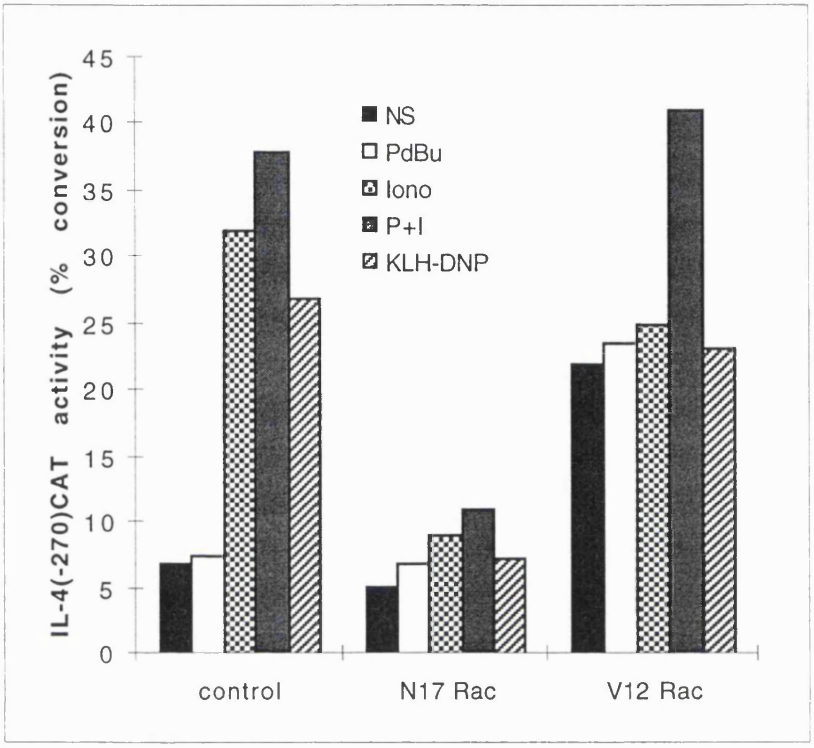


Figure 5.7. The Rac-1 GTPase is involved in FcεR1 induction of IL-4 (-270) CAT activity in RBL2H3. 10^7 RBL2H3 per point were transfected with 20μg reporter alone or in combination with an equivalent amount of the pEF N17 Rac-1 or pEF V12 Rac-1. Cells were recovered for 6h before IgE priming and stimulation as indicated. Concentrations of stimuli used were as follows; Ionomycin, 500ng/ml; PdBu, 50ng/ml; KLH-DNP, 500ng/ml. After 16h stimulation cells were harvested and CAT activity was assayed. Data are representative of three experiments.

The ability of V12 Ras to strongly induce IL-4 (-270) CAT activity in the absence of other stimuli suggests that Ras effector pathways are involved in FcεR1 regulation of the IL-4 promoter region. The inhibition of IL-4 (-270) CAT activity observed when PD 098059 is applied initially suggested that the Raf1-/MEK pathway may be responsible in a manner analogous to that observed for FcεR1 regulation of Elk-1. However, Raf-1 CAAX could not induce IL-4 (-270) CAT activity in the absence of other stimuli or potentiate FcεR1 induction of the reporter. These data suggest that activation of the Raf-1/MEK signalling pathway is not sufficient for induction of the IL-4 (-270) CAT reporter gene. However, there is clearly a requirement for this signalling pathway since inhibition of the central MEK kinase consistently impaired FcεR1 regulation of IL-4 (-270) CAT.

The data presented so far show that it is possible to measure FcεR1 mediated induction of a reporter gene of the 270bp proximal to the TATA box of the murine IL-4 promoter. The use of dominant inhibitory mutants in cotransfection assays suggests that there are targets in this region for both Ras and Rac-1 derived signals. However, the IL-4 (-270) region is not straightforward to work with since 1) there is a high basal activity of this reporter gene in RBL2H3, 2) the fold increases in induction observed with either pharmacological or FcεR1 stimulations are low and 3) the large amounts (>25μg) of reporter gene DNA required mean that co-transfection experiments approach the limits of DNA tolerance for the RBL2H3 in transient transfection by the method employed here. In order to progress further, the murine IL-4 promoter was examined and by analogy with T cell antigen receptor regulation of the IL-2 and IL-4 promoters, a critical regulatory element was identified and studied.

5.2.3 NFAT/AP-1 transcription factor complexes are regulated by the FcεR1 in RBL2H3.

T cell antigen receptor induction of the genes for IL-2 and IL-4 is dependent on the activity of a wide number of transcription factors. However, numerous studies have assigned a critical regulatory role to the transcription factor complex comprising a member of the Nuclear Factor of Activated T cells (NFAT) family and a homo- or hetero-dimer of AP-1 family proteins. Current models for NFAT/AP-1 complex regulation will be discussed in detail in the introduction to Chapter 6. However, it is clear that calcium, Ras and Rac-1 derived signals are all capable of regulating components of the NFAT/AP-1 complexes. Since these are the signals which are apparently targeting elements of the IL-4 promoter in RBL2H3, an assay for NFAT/AP-1 transcriptional activity was established in RBL2H3. The rationale for these experiments was to examine the signalling requirements for FcεR1 regulation of NFAT/AP-1 complexes and hence focus upon one critical regulatory element from the many contained in the whole IL-4 promoter region.

Initial experiments established NFAT/AP-1 reporter gene assays in RBL2H3. In this system, trimerised NFAT/AP-1 sites from the IL-4 gene promoter were used to drive CAT reporter gene activity. Oligomerisation of nucleotide sequences upstream of reporter genes is a generally applied method which introduces some amplification into the reporter system. The sequence of the NFAT/AP-1 site used (derived from the IL-4 promoter) is shown in Figure 5.8.

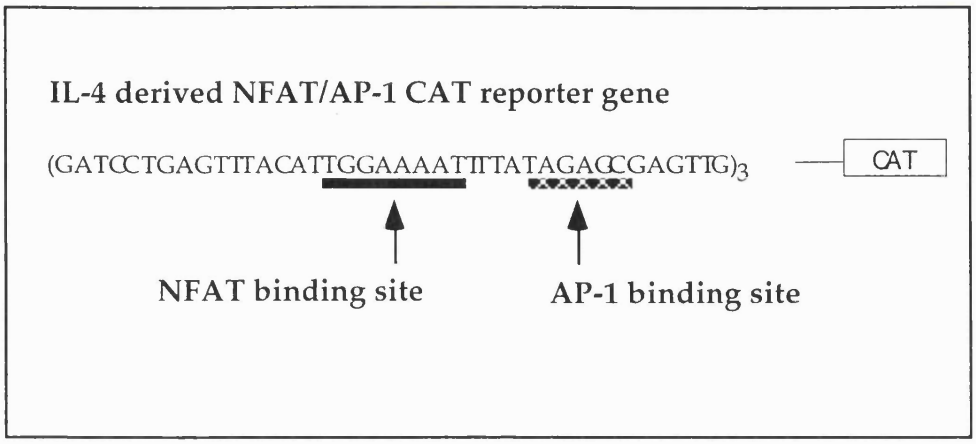


Figure 5.8. IL-4 NFAT/AP-1 reporter gene construct. An oligonucleotide comprising trimerised versions of the NFAT/AP-1 site from Purine Box B of the murine IL-4 promoter region was spliced to the Chloramphenicol Acetyltransferase (CAT) gene (Chuvpilo *et al.*, 1993). NFAT, nuclear factor of activated T cells; AP-1, activator protein-1; IL, interleukin.

Initial experiments established that the NFAT/AP-1 reporter gene construct shown in Figure 5.8 could be used in transient transfection assays in the RBL2H3. The data in Figure 5.9 show the effect of pharmacological stimulation of NFAT/AP-1 CAT activity . In RBL2H3 phorbol esters alone are not able to significantly induce the activity of the IL-4 NFAT/AP-1 CAT reporter. Elevation of intracellular free calcium levels using the ionophore Ionomycin is able to induce activity of IL-4 NFAT/AP-1 CAT in a dose dependent manner. Ionomycin alone is sufficient for induction of the IL-4 derived NFAT/AP-1 reporter in RBL2H3 and no potentiation is observed with addition of phorbol ester. These data suggest that the transcription factor complex binding to the IL-4 NFAT/AP-1 site in RBL2H3 is highly sensitive to calcium signals. Protein kinase C signals which are induced by PdBu cannot induce IL-4 NFAT/AP-1 CAT activity. The maximal stimulation of reporter activity observed with Ionomycin stimulation means that any synergy which may be observed with combination of PdBu and Ionomycin is likely to be masked. However, when Ionomycin concentrations were titrated to sub-optimal levels there was still no observable synergy between the two stimuli.

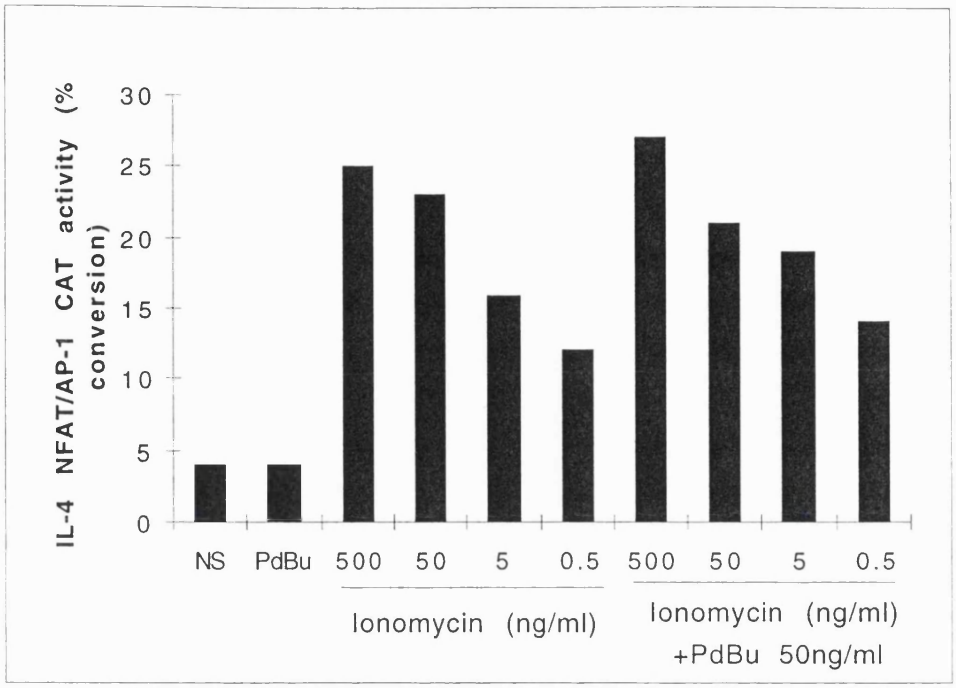


Figure 5.9. Phorbol ester and Ionomycin stimulation of NFAT/AP-1 CAT activity in RBL2H3. 10^7 RBL2H3 per point were transfected with 15μg IL-4 derived NFAT/AP-1 reporter and recovered for 1h before stimulation with the indicated concentrations of ionomycin alone or in combination with 50ng/ml PdBu. After 16h stimulation cells were harvested and CAT activity was assayed. Data are representative of three experiments.

In order for FcεR1 regulation of NFAT/AP-1 sites to be examined the reporter gene was transfected into RBL2H3 which were then exposed to IgE/antigen. The data in Figure 5.10 show that the IL-4 derived NFAT/AP-1 reporter gene is regulated by the FcεR1 in a manner dose dependent on the amount of crosslinking antigen applied. The typical antigen receptor dose response shown here indicates that 50% maximal stimulation is achieved at approximately 20ng/ml KLH-DNP. These data establish that regulation of NFAT/AP-1 complexes in RBL2H3 may be studied using the IL-4 NFAT/AP-1 CAT reporter.

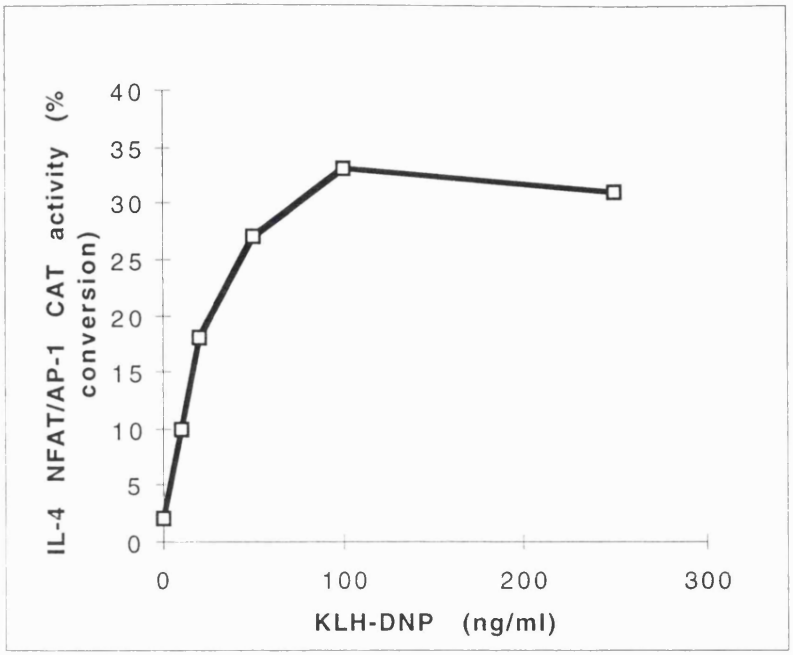


Figure 5.10. FcεR1 stimulation of NFAT/AP-1 CAT activity in RBL2H3. 10^7 RBL2H3 per point were transfected with 15μg IL-4 derived NFAT/AP-1 reporter and recovered for 1h before IgE priming and stimulation with the indicated concentrations of KLH-DNP. After 16h stimulation cells were harvested and CAT activity was assayed. Data are representative of three experiments.

5.2.3 NFAT/AP-1 transcription factor complexes may be activated by Ras and calcineurin signals in RBL2H3.

As will be discussed in detail in Chapter 6, NFAT/AP-1 complexes in the T cell are known targets for Ras and calcium derived signals. The data in Figure 5.9 have shown that pharmacological delivery of a calcium signal strongly activates the IL-4 derived NFAT/AP-1 site. It is likely that, as in the T cell, a major target for this calcium signal lies in the activity of the calcium dependent phosphatase Calcineurin (CN). CN is responsible for the dephosphorylation of cytosolic NFAT protein, a step permissive for NFAT nuclear import. Without this import step cytosolic NFAT protein does not come into contact with DNA. NFAT/AP-1 transcriptional activity in T cells is absolutely dependent on CN activity. T cells treated with the macrolide immunosuppressant drug Cyclosporin A, which selectively inhibits Calcineurin, cannot activate NFAT/AP-1 complexes or produce IL-2 or IL-4. T cell antigen receptor activation of NFAT/AP-1 complexes also requires Ras activity, this latter signal is thought to target the AP-1 component of the NFAT/AP-1 complex. There is extensive published data showing that Ras and calcium signals in fact synergistically activate NFAT/AP1 transcription complexes in T cells in the context of the IL-2 promoter (Genot *et al.*, 1996; Woodrow *et al.*, 1993). Co-transfection experiments

next addressed the effect of activated mutants of Ras and the calcium regulated phosphatase Calcineurin upon NFAT/AP-1 reporter gene activity.

The data in Figure 5.11 show the effect of cotransfection of activated mutants of Ras (V12Ras) or Calcineurin (CNM) alone or in combination on the activity of the IL-4 NFAT/AP-1 CAT reporter gene. The activated Calcineurin mutant alone strongly induced NFAT/AP-1 activity by approximately 5 fold over background levels. Co-transfection with the activated V12 Ras alone weakly stimulated NFAT/AP-1 CAT activity with a 1.5-2 fold induction. When V12 Ras and CNM were used in combination, the effect of the two mutants was additive. Titration of the levels of CNM used did not reveal any synergy between activated Ras and CNM signals even at low levels of CNM expression.

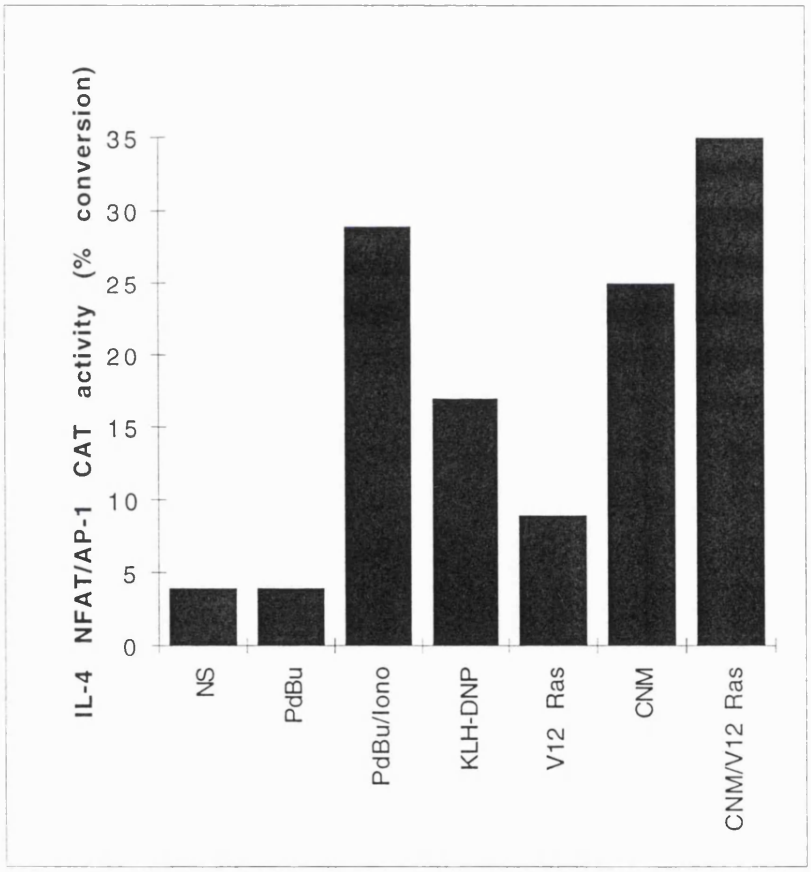
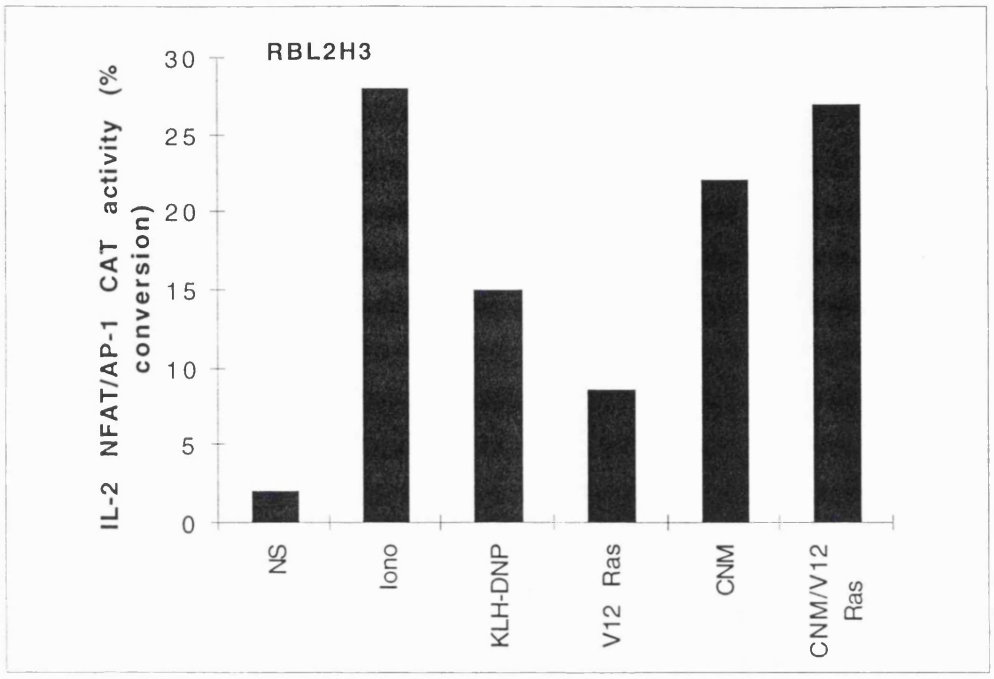


Figure 5.11. Effects of activated mutants of Ras and Calcineurin regulates NFAT/AP-1 CAT activity in RBL2H3. 10^7 RBL2H3 per point were transfected with 15μg IL-4 derived NFAT/AP-1 CAT reporter alone or in combination with 20μg pEF V12 Ras or 5μg pSRα CNM and recovered for 6h before stimulation with the indicated concentrations of ionomycin alone or in combination with 50ng/ml PdBu, or IgE priming and stimulation with 500ng/ml KLH-DNP. After 16h stimulation cells were harvested and CAT activity was assayed. Data are representative of three experiments.

The IL-4 NFAT/AP-1 CAT reporter is maximally induced either by the application of calcium ionophore or co-transfection of the activated CN mutant. Moreover, Ras and calcium signals do not synergise for activation of the IL-4 derived NFAT/AP-1 complex. These data are in contrast with the reported regulation of the IL-2 gene derived NFAT/AP-1 complex in the T cell system, where a clear synergy between Ras and calcium signals is observed. The question arises of whether this reflects 1) a genuine difference between regulation of NFAT/AP-1 complexes in the two cell types or 2) a difference in the regulation of the two distinct IL-2 and IL-4 derived reporter genes.

In order to test this point the IL-2 NFAT/AP-1 reporter gene (represented schematically in Figure 5.13) was used in the RBL2H3 cell line. A comparison was performed with the regulation of this construct in the Jurkat T cell line. These data are shown in Figure 5.12. In the RBL2H3 (Figure 5.12a) the effect of either calcium ionophore or the activated mutant of CN (CNM) was to maximally induce IL-2 NFAT/AP-1 CAT activity. Co transfection of the activated V12 Ras mutant did not enhance this induction. In contrast, a similar experiment in the Jurkat T cell line shows that calcium or CN signals are not sufficient to induce IL-2 NFAT/AP-1 CAT activity to maximal levels (Figure 5.12b). In this cell system, Ras and calcium signals synergise for full induction of the reporter. These data suggest that in fact NFAT/AP-1 sites are differently regulated by calcium signals in the RBL2H3 and Jurkat T cell systems.

a



b

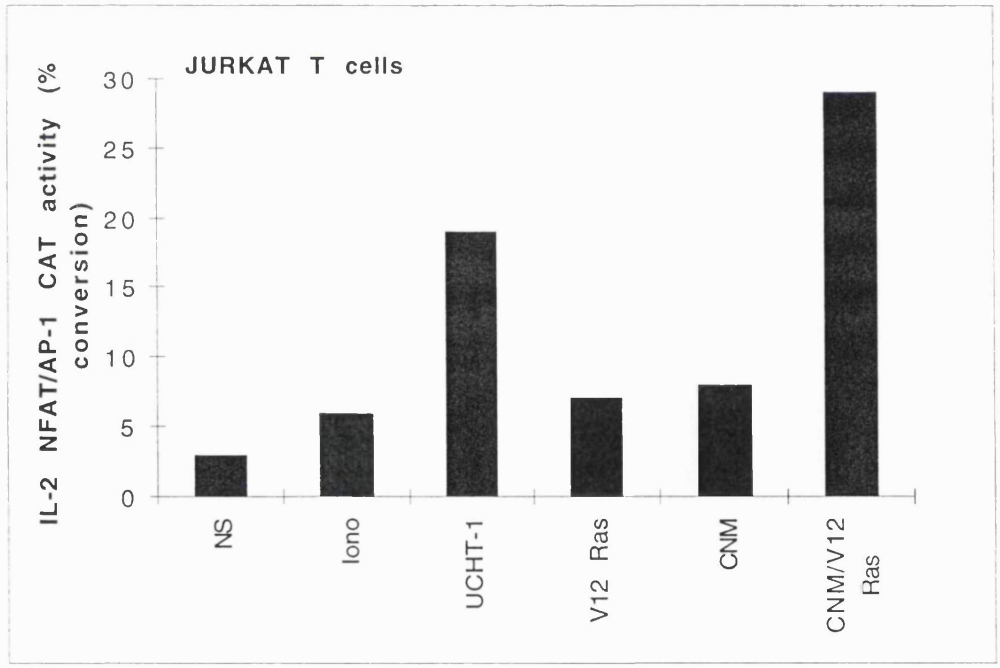


Figure 5.12. Regulation of IL-2 derived NFAT/AP-1 CAT reporter genes in RBL2H3 and JURKAT T cells 10^7 RBL2H3 or JURKAT T cells per point were transfected with 15μg IL-2 derived NFAT/AP-1 CAT reporter alone or in combination with 20μg pEF V12 Ras or 5μg pSRα CNM and recovered for 6h before stimulation with the indicated reagents. Concentrations of stimuli were 500ng/ml Ionomycin, 500ng/ml KLH-DNP or 2μg/ml UCHT-1 (TCR stimulatory antibody). After 16h stimulation cells were harvested and CAT activity was assayed. Data are representative of three experiments.

5.2.4 NFAT and AP-1 binding are required for activity of the NFAT/AP-1 complex in RBL2H3, although AP-1 does not transactivate independently.

The FcεR1 regulates NFAT/AP-1 transcriptional complexes. Activated mutants of both Ras and the CN phosphatase can induce activity of an NFAT/AP-1 reporter gene in the absence of other stimuli. Conventional models derived on the basis of data from the T cell system suggest that Ras signals target AP-1 dimers and CN activation results in nuclear import and hence activity of the NFAT protein. Assembly of the NFAT/AP-1 complex is thought to require interactions of NFAT and both AP-1 proteins with DNA. However, not all AP-1 regulated transcriptional events occur in the context of an NFAT interaction. It is established that in the T cell AP-1 proteins can independently transactivate and drive the expression of a reporter gene construct. The roles of the NFAT and AP-1 factors in transcriptional activation in mast cells were examined as follows.

A set of CAT reporter gene constructs were obtained from Dr Anjano Rao, Dana Farber Cancer Institue, Boston. These comprised trimerised verions of 1) a wild-type NFAT/AP-1 site derived from the human IL-2 promoter and 2) two point mutated verions of this construct in which either the NFAT (M3) or AP-1 (M4) site had been disrupted (Jain *et al.*, 1995a). These constructs were used in RBL2H3 to examine whether both components of the complex are absolutely required for transcriptional activity.. The sequences of these mutant reporters are shown schematically in Figure 5.13. In addition, a trimerised AP-1 site was used to drive expression of a CAT reporter to ask if AP-1 dimers could independently transactivate in the RBL2H3 system.

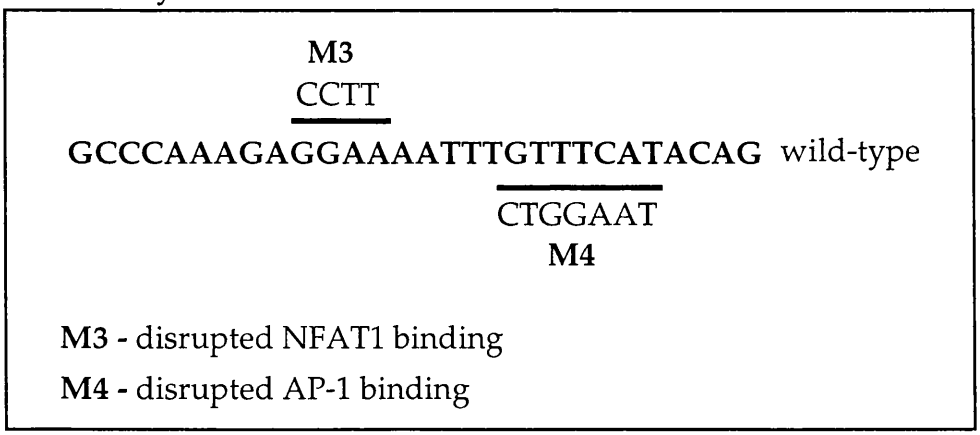


Figure 5.13. NFAT/AP-1 oligonucleotides used to construct wild-type and mutated reporter gene constructs. Base substitutions made to the sequence of the NFAT/AP-1 site derived from the human IL-2 promoter are shown. The resulting oligonucleotides were used in a trimerised form to drive expression of a CAT reporter gene. The M3 and M4 designation of compromised NFAT1 and AP-1 binding sites respectively is from (Jain *et al.*, 1995a) where the constructs were first described.

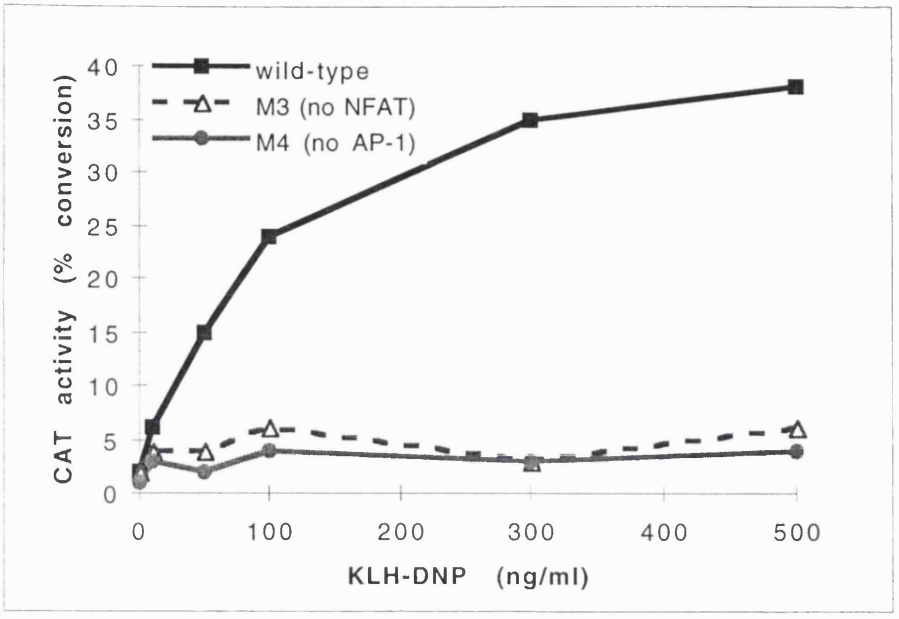
Wild-type, M3 and M4 NFAT/AP1- sites were transiently transfected into RB2H3 and their induction by the FcεR1 was measured. The data in Figure 5.14a show that only the wild-type (WT) NFAT/AP1 site is inducibly regulated by the FcεR1. Disruption of either the NFAT1 binding site (M3) or the AP-1 binding site (M4) results in the loss of regulation of the reporter gene by the FcεR1. It is clear that disruption of either site results in a total loss of function. These data suggest that both NFAT and AP-1 proteins are absolutely required for induction of reporter gene activity. Hence a productive complex between the two factors is required for transactivation.

A CAT reporter gene driven by a trimerised AP-1 site was used in RBL2H3 to examine whether AP-1 dimers are able to independently recruit the basal transcriptional machinery and induce reporter gene activity. The data in Figure 5.14b show that neither antigenic stimulation of the FcεR1 or the application of PdBu is sufficient to induce AP-1 CAT transcriptional activity in RBL2H3. In contrast, in the T cell system (Figure5.14c), both ligation of the T cell antigen receptor and PdBu can strongly induce AP-1 CAT activity.

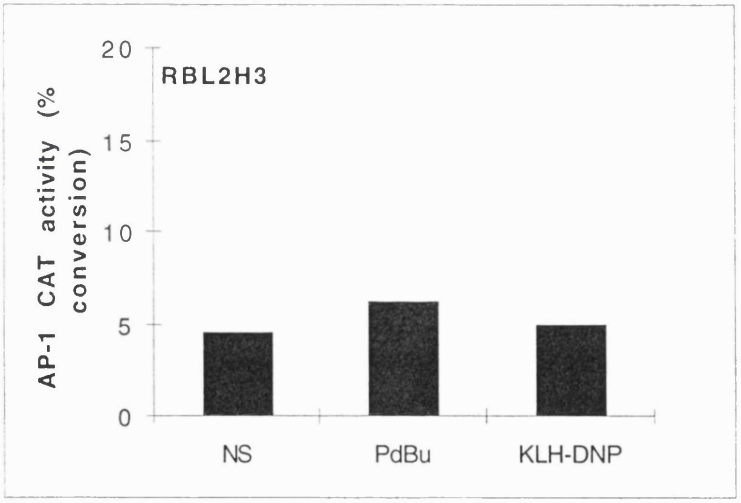
Figure 5.14. NFAT and AP-1 act co-operatively to induce CAT reporter gene activity. 10^7 RBL2H3 per point were transfected with a) 15μg of the indicated reporter and recovered for 1h before priming and stimulation with the indicated concentrations of KLH-DNP. b) 10μg of AP-1 CAT reporter, cells were recovered for 1h before priming and stimulation with 500ng/ml KLH-DNP or treatment with 50ng/ml PdBu alone. c) 10^7 Jurkat T cells per point were transfected with 10μg of AP-1 CAT reporter, cells were recovered for 1h before stimulation with either 50ng/ml PdBu or TCR ligation using 2μg/ml UCHT-1. All cells were stimulated for 16h before harvesting and CAT assay as described.

Figure 5.14

a



b



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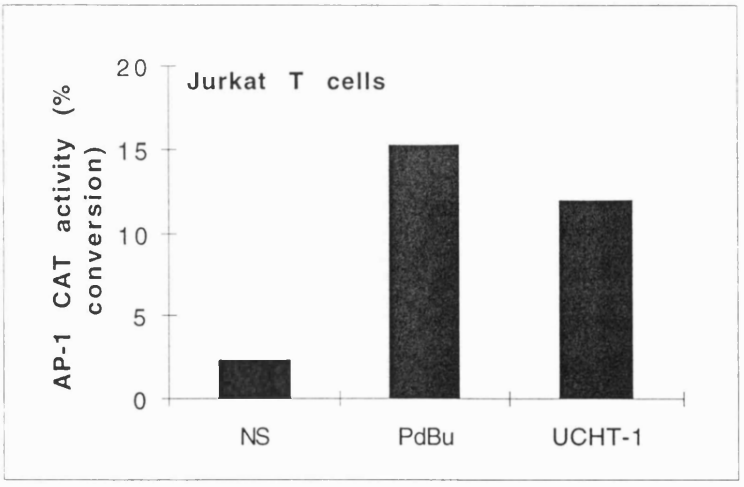


5.3 DISCUSSION.

5.3.1 Calcium, Ras and Rac-1 signals can regulate the IL-4 (-270) promoter region in RBL2H3.

The data presented in this Chapter have established two types of CAT reporter gene assay for elements of cytokine gene regulation in RBL2H3 mast cells. First, a 270 bp region of the murine IL-4 promoter was used to drive CAT reporter gene expression. The data show that calcium ionophore or FcεR1 stimulation via IgE/antigen can induce activity of the IL-4 (-270) CAT reporter gene. The IL-4 (-270) region is strongly calcium regulated and does not display enhanced activity when stimulated with ionomycin in combination with phorbol ester.

It is clear from the data presented in Figures 5.5 and 5.7 that the induction of IL-4 (-270) CAT activity is sensitive to the action of dominant inhibitory mutants of both Ras and Rac-1. These data strongly suggest that the ability of the FcεR1 to regulate this region of the IL-4 promoter is dependent on the activity of these GTPases. In accord with this suggestion is the observation that if either of these GTPases are used in a constitutively active form, then they are able to activate IL-4 (-270) CAT in the absence of other stimuli. Investigation of the relevant Ras effector pathway for FcεR1 regulation of IL-4 (-270) CAT was attempted. These experiments used a selective inhibitor of the MEK kinase and an activated form of Raf-1 in order to ascertain whether inhibition or activation respectively of the Raf-1/MEK/ERK Ras effector cascade affected FcεR1 induction of the IL-4 (-270) CAT reporter.

The MEK inhibitor PD 098059 markedly inhibited FcεR1 induction of the whole IL-4 promoter. However, the activated Raf-1 CAAX mutant, proposed to drive the Raf-1/MEK/ERK cascade by providing a constitutive signal to endogenous MEK, failed to induce CAT activity or to potentiate that observed in FcεR1 stimulated cells. These data suggest that Raf-1/MEK signals are necessary but not sufficient for FcεR1 induction of IL-4 (-270) CAT activity. Calcium, Ras and Rac-1 derived signals contribute to FcεR1 regulation of this promoter region in RBL2H3. The FcεR1 signals thought to regulate this promoter region are shown schematically in Figure 5.15.

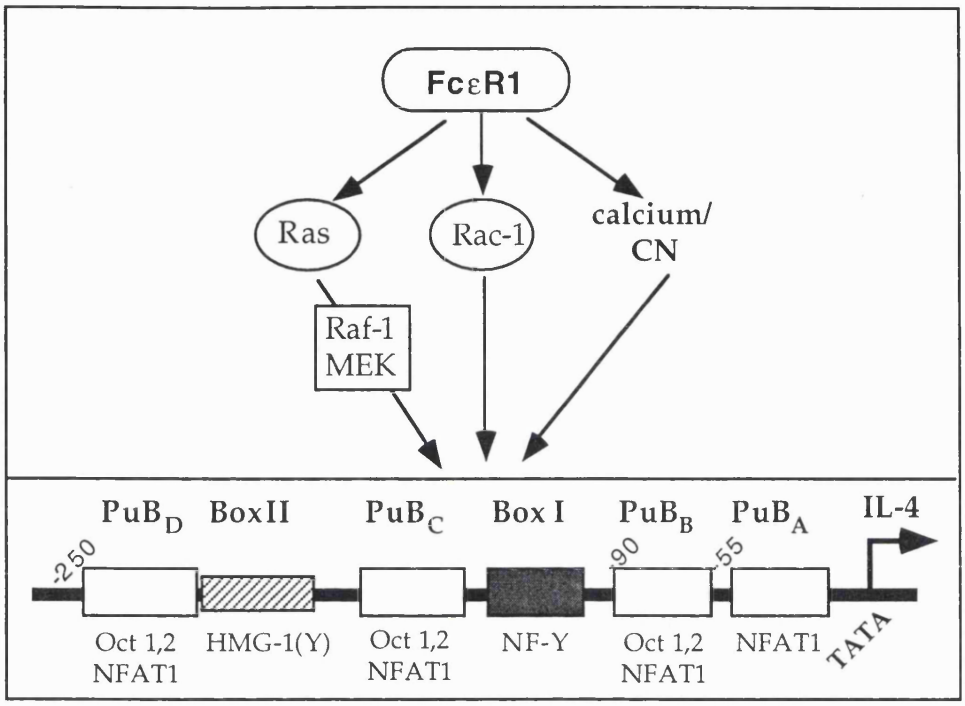


Figure 5.15. Multiple FcεR1 signals target the IL-4 promoter region in mast cells. The IL-4 promoter is regulated by the FcεR1. Signals which affect this region in RBL2H3 include the Ras family GTPases Ras and Rac-1 and a calcium/Calcineurin (CN) pathway. The multiple regulatory elements of the IL-4 promoter are defined in the legend to Figure 5.1.

There are a number of technical problems inherent in the use of the IL-4 (-270) CAT construct to assign roles to particular signalling pathways. 1) The low fold inductions and high basal reporter gene activity observed in the RBL2H3 using IL-4 (-270) CAT mean that assays such as antigen dose responses are not possible. When partial effects of inhibitors or inhibitory GTPase mutants are being mapped, extensive antigen dose responses are necessary to ensure the validity and significance of the data. 2) The assay itself is unwieldy. The amount of reporter DNA needed is in excess of 25μg/10^7 RBL2H3. This requirement severely limits the possibility of co-transfection with plasmids encoding mutant signalling molecules. When the amount of DNA used to transfect RBL2H3 by electroporation exceed 40μg, the transfection efficiencies obtained drop dramatically and it is not possible to use an excess of regulator plasmid over reporter in order to ensure that any cell transfected with reporter gene has the best statistical chance of also bearing the regulatory molecule of interest.

In addition to the technical issues described above, IL-4 (-270) CAT may not be a valuable reporter to model behaviour of the endogenous IL-4 promoter. In RBL2H3, the IL-4 (-270) CAT gene has a high basal level of activity and is only weakly induced by the FcεR1. The situation expected for the endogenous IL-4 gene

would be a very low basal induction and high levels of FcεR1-induced IL-4 promoter activity.

5.3.2 Regulation of NFAT/AP-1 reporter gene by FcεR1 derived signals in RBL2H3.

The whole IL-4 promoter, like the IL-2 promoter-enhancer region, contains multiple binding sites for regulatory transcription factors. These regulatory regions will contribute in differing degrees to the activity of a reporter gene driven by the whole promoter region. As described above, the regulation of the whole IL-4 promoter region is difficult to study in detail. However, in the case of the IL-2 and IL-4 gene promoters in the T cell system, it is known that assembly of a productive, transactivating NFAT/AP-1 complex is absolutely required for promoter activity. These transcriptional complexes are therefore known as critical determinants of cytokine gene induction in a number of systems. The regulation of NFAT/AP-1 complexes in RBL2H3 was therefore examined.

The data presented in Figure 5.10 show that the FcεR1 is capable of regulating reporter gene activity driven by trimerised NFAT/AP-1 sites derived from the murine IL-4 promoter. These data suggest that this system may well prove to be a sensitive assay for the role of signalling molecules such as GTPases in FcεR1 regulation of cytokine gene induction. The data to be presented in Chapters 6 and 7 show detailed analyses of IL-4 NFAT/AP-1 regulation by the FcεR1 in RBL2H3 mast cells.

Calcium signals delivered by ionophore or mimicked by a constitutively active Calcineurin are sufficient for full induction of the NFAT/AP-1 reporter. Addition of agents such as phorbol ester or IgE/antigen does not enhance this induction. In RBL2H3 mast cells, the IL-4 NFAT/AP-1 site does not behave in a manner strongly reminiscent of its T cell counterpart, the IL-2 derived NFAT/AP-1 site. In the latter case, Ras and calcium signals are needed to synergise for full induction of the NFAT/AP-1 complex. The IL-2 derived NFAT/AP-1 site is strongly calcium regulated in RBL2H3. This suggests that the requirements for regulation of NFAT/AP-1 sites differ between T cells and RBL2H3, rather than reflecting a difference in the reporter genes themselves.

Calcium signals delivered by the application of ionophore or mimicked by the introduction of a constitutively active calcineurin mutant are sufficient to maximally induce NFAT/AP-1 activity in RBL2H3. These data do not necessarily reflect that the only FcεR1 signal important in this context is the elevation of intracellular free calcium levels. Both the manipulations with CN and ionophore are essentially pharmacological, providing a signal which may differ both quantitively and/or qualitatively from that induced by the FcεR1. FcεR1 regulated signalling pathways which regulate the NFAT/AP-1 reporter gene are examined in detail in Chapter 6.

NFAT and AP-1 proteins act cooperatively to form an NFAT/AP-1 transcriptional complex. Disruption of binding of one or other of the components results in loss of function. These data are born out for the RBL2H3 system, where it is found that AP-1 proteins themselves cannot transactivate and induce CAT reporter gene activity. These data are in contrast with the T cell system where AP-1 CAT activity is induced by both phorbol esters and ligation of the TCR. While AP-1 does not transactivate in the mast cell system, this would suggest that AP-1 protein are required in a primarily architectural role in the context of IL-4 promoter derived NFAT/AP-1 complexes.

The data presented at the end of this Chapter have established that FcεR1 regulation of an NFAT/AP-1 reporter gene derived from the murine IL-4 promoter can be sensitively assayed in RBL2H3. This assay system was deemed to have a number of advantages over a reporter driven by the whole IL-4 promoter. Subsequent experiments were directed towards a detailed analysis of the signalling pathways used by the FcεR1 to regulate this transcription factor complex. These experiments are presented in Chapter 6 and 7.

CHAPTER SIX

The role of Ras family GTPases in the FcεR1 regulation of NFAT/AP-1 transcriptional complexes.

6.1 INTRODUCTION.

NFAT/AP-1 transcriptional complexes regulate a variety of genes which are pivotal to the development of an immune response (Rao *et al.*, 1997). As targets for the immunosuppressive drugs Cyclosporin A and FK506, NFAT proteins have been extensively studied. In the context of the mast cell system, NFAT/AP-1 complexes are likely to be important targets of FcεR1 signals. The data in Chapter 5 have shown that a reporter gene driven by a trimerised NFAT/AP-1 site derived from the IL-4 promoter is regulated by the FcεR1, suggesting that NFAT/AP-1 activation is a consequence of mast cell activation. The regulation of NFAT/AP-1 complexes in the T cell system has been examined in some detail, resulting in the identification of a number of T cell antigen receptor derived signalling pathways which target NFAT/AP-1 in the context of the IL-2 promoter. These include elevation of intracellular free calcium and the activity of the Ras family GTPases Ras and Rac-1 (Genot *et al.*, 1996; Woodrow *et al.*, 1993). The data presented in this Chapter examine the role of Ras family GTPases in FcεR1 control of NFAT/AP-1 activity.

6.1.1 The NFAT family of transcription factors.

Transcription factors of the NFAT family are widely distributed in a variety of immune and non-immune system tissues. heir properties are reviewed in detail in (Rao *et al.*, 1997). Four major NFAT isoforms (NFAT1-4) are cloned, within classes 1,2 and 4 there are splice variants such that a total of 10 NFATs are now known. Schematic primary structures for the NFAT family are shown in Figure 6.1. Literature on the cloning and isolation of NFAT family members is summarised in Figure 6.2. Several regions of homology characterise NFAT proteins. The NFAT homology region (NHR) is a regulatory region of the NFAT protein comprising approximately three hundred residues N-terminal to the DNA binding domain. Truncation experiments show that this is the region of NFAT responsible for interaction with the Calcineurin phosphatase (Luo *et al.*, 1996b) This region in NFAT1 contains phosphorylated Serine residues which have been shown to be dephosphorylated by CN *in vitro*. The regulatory interaction with CN and the role of these Ser phosphoacceptor sites is discussed in more detail in the introduction to Chapter 7.

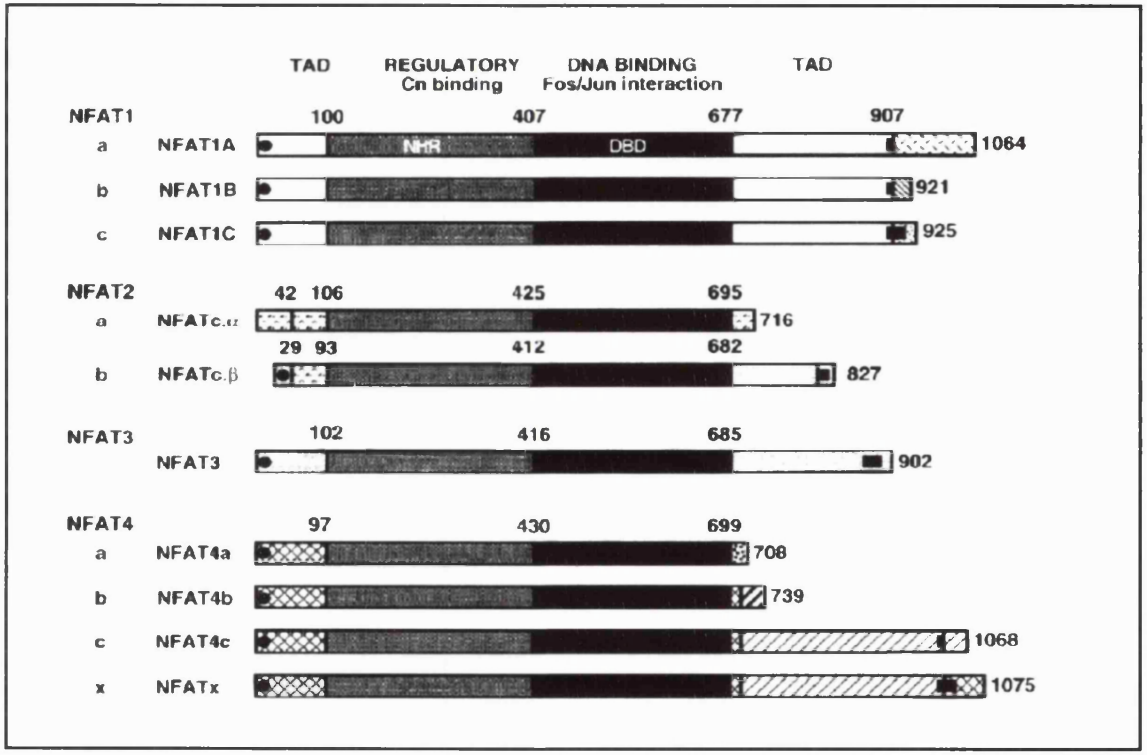


Figure 6.1 The NFAT family of transcription factors. Reproduced from Rao *et al*, Annual Review of Immunology, 1997. NFAT proteins are an extensive transcription factor family. Highly homologous regions are the regulatory NFAT homology region (NHR) and the Rel homology DNA binding domain (DBD). NFAT proteins contain proposed N- and C-terminal transactivation domains (TADs), the former containing acidic/hydrophobic motifs (●). The black C-terminal bars represent the LDQTYLDDVNEIIRKEFS sequence which may enhance the transactivation potential of an isoform. Amino acid numbers refer to the human proteins.

Isoform	Protein purified	cDNA library	Reference
NFAT1A NFAT1B NFAT1C	Ar-5 murine T cells	Jurkat humanT cells	Jain *et al* 1993 McCaffrey *et al* 1995
NFAT2A NFAT2B	Bovine thymus Raji B cells	Jurkat humanT cells	Northrop *et al* 1994 Park et al 1996
NFAT3	Jurkat humanT cells	peripheral blood T cell	Hoey *et al* 1995
NFAT4A NFAT4B NFAT4C	Jurkat humanT cells	peripheral blood T cell	Hoey *et al* 1995
NFAT4X	bovine thymus		Masuda et al 1995 Ho et al 1995

Figure 6.2. Cloning and isolation of NFAT family members. Sources of purified NFAT family members and cDNA libraries from which they were cloned are indicated, with relevant references (Ho *et al.*, 1995; Hoey *et al.*, 1995; Jain *et al.*, 1993; Masuda *et al.*, 1995; McCaffrey *et al.*, 1993; Northrop *et al.*, 1994; Park *et al.*, 1996)..

The DNA binding domain of NFAT proteins is similar to that found in the Rel family of transcription factors. The minimal DNA binding domain of NFAT1 is approximately 200 amino acids and closely resembles the specificity determinant region of the Rel-family NF-κB p50 subunit. This region in NFAT contains the recognition loop which is conserved in the Rel family, **RAHYETEG**, where the bolded residues are thought to contact DNA. A major difference between NFAT and Rel family transcription factors is that NFAT proteins bind DNA as monomers rather than the obligate dimers formed by Rel. Instead, NFAT proteins bind DNA in the context of a cooperative interaction with a dimer of AP-1 proteins. The interaction with AP-1 involves sequence specific binding of the two factors to adjacent DNA sites. NFAT/AP-1 cooperation results in a 20-fold increase in the stability of the transcription complex: DNA interaction compared with the interaction of DNA with NFAT1 alone (Jain *et al.*, 1995a). The most well characterised NFAT/AP-1 interaction is that of NFAT1 with a Fos/Jun AP-1 heterodimer. In this complex, the AP-1 half-site which is closest to the NFAT1 protein selects exclusively for Jun. Hence there is an orientational specificity for NFAT1-Jun-Fos (5'-3') (Chen *et al.*, 1995).

Cooperative interactions with AP-1 have been described for the majority of genetic contexts where NFAT binding sites are present. This type of transcriptional cooperativity implies that transcriptional transactivation (binding to DNA and recruitment of the transcriptional machinery) is either dependent upon or enhanced by

the interaction. There is evidence that certain regions of NFAT (best studied in the case of NFAT1) can function as transactivation domains in their own rights. These are stretches of acidic/hydrophobic residues which have can drive transactivation if experimentally fused to, for example, a Gal4 DNA binding domain (DBD) (Luo *et al.*, 1996a). The significance of NFAT intrinsic transactivational ability is not completely clear. The data could reflect 1) that NFAT can independently recruit the transcriptional machinery but with a low affinity which is not physiologically relevant unless AP-1 is present, 2) that NFAT does recruit the transcriptional machinery to NFAT/AP-1 complexes *in vivo* and the role of AP-1 is to stabilise the complex or 3) that the intrinsic transactivational ability of NFAT is relevant to, as yet uncharacterised, situations where NFAT functions in isolation or in the context of a non-AP-1 binding partner. Thus the significance of the NFAT acidic/hydrophobic transactivation motifs is not yet understood.

6.1.2 NFAT target genes are important components of the immune response.

There are a large number of potential target genes for NFAT transcription factors. The data upon roles for NFAT in regulation of these genes can be divided into two classes. Firstly, for some genes NFAT involvement has been established convincingly by experiments such as mutation of an NFAT site decreasing expression of a reporter driven by a particular promoter, overexpression of NFAT protein driving reporter expression or multimerisation of a site being used to confer sensitivity to Cyclosporin A upon induction of a reporter gene. NFAT regulated genes in this class are as follows; IL-2, IL-4, GM-CSF, TNFα, IL-3, IL-5, IL-8, IFNγ and the CD40-ligand molecule. An involvement for NFAT in regulation of other target genes has been inferred from the sensitivity of gene expression to the immunosuppressive drugs Cyclosporin A or FK506. These compounds target the CN phosphatase, upon which NFAT activity is dependent, but may have other intracellular targets or be non-specific at high doses. Genes in this second class include IL-1β, IL-10, IL-13, TGF-β, CD25, CD69, CTLA-4, Fas-ligand and the transcription factors NF-κB, c-Rel, Oct-2, NFAT2 and Nur-77. References for the individual studies on each of these target genes may be found in (Rao *et al.*, 1997) .

There is clearly enormous potential for NFAT transcription factors to modulate multiple aspects of the immune reponse. There is possible functional redundancy between isoforms of this extensive protein family. Taken together, these facts complicate examination of NFAT function *in vivo*. This is demonstrated by the data from experiments where mice were generated which were deficient in the NFAT1 isoform. Two groups performed these analyses (Hodge *et al.*, 1996; Xanthoudakis *et al.*, 1996), generating mice expressing a deletion mutant of NFAT1 without DNA

binding activity or with a null mutation respectively. The mice developed normally and showed no major deficiency in levels of IL-2, IL-4, TNFα or IFNγ which could be produced by isolated spleen cells. The simplest interpretation if these data suggests that *in vivo*, NFAT1 is not required for production of these cytokines. However, there is considerable evidence that NFAT family members bind DNA with a degree of redundancy. Hence, NFAT1 deficiency may be largely compensated for by other NFAT isoforms. Interestingly, certain aspects of immunity were enhanced in NFAT1 deficient mice. Th2 type responses were enhanced in three separate assays, these responses are thought to be dependent on IL-4 for their early and late phases. Hence in NFAT deficient mice it might have been expected that these responses would be compromised. These data reflect the complexity of NFAT dependent transcription in the immune response. Deficiency in such a pleiotropic factor is not affecting only one aspect of an immune response. Multiple compensatory mechanisms may be at work and the primary genes responsible for the observed phenotype may be NFAT targets normally involved in negative immune regulation. While NFAT regulation of genes such as those for IL-2 and IL-4 are the most studied *in vitro*, this is not necessarily a reflection of their importance in generation of the phenotype of NFAT1 deficient mice.

6.1.3 Regulation of NFAT/AP-1 complexes in T and mast cells.

NFAT/AP-1 complexes have been most intensively studied in the T lymphocyte system. The IL-2 and IL-4 promoter regions both contain multiple composite NFAT/AP-1 sites which can drive expression of a heterologous reporter gene. The signalling pathways which regulate NFAT/AP-1 sites have been examined in an experimental system consisting of such a reporter gene, driven by trimerised IL-2 gene derived NFAT/AP-1 sites. Data from this experimental system on signalling pathways has not been significantly extended by examination of the IL-4 NFAT/AP-1 site in either T cells or mast cells.

Current models suggest that two T cell antigen receptor derived signals are critically important for the transcriptional activation of NFAT/AP-1. It is recognised that these signals must induce two distinct downstream events; 1) NFAT, which is basally phosphorylated in resting cells, must be dephosphorylated. The de-phosphorylated NFAT can move to the nucleus where it is able to interact with DNA. 2) The AP-1 binding partner for NFAT must be synthesised, typically the promoters for immediate early genes such as Fos and Jun must be induced. Data from studies of TCR regulated IL-2 NFAT/AP-1 CAT reporter genes allow a simple two signal model to be proposed for TCR induction of these twin events.

TCR controlled elevation in intracellular free calcium acts to relieve autoinhibition of the calcineurin (CN) phosphatase (Kissinger *et al.*, 1995). This phosphatase is the target of the immunosuppressive drugs Cyclosporin A and FK506,

which have been used to show that activity of NFAT/AP-1 sites and dephosphorylation of NFAT protein are dependent upon CN. TCR regulation of intracellular free calcium therefore controls the phosphorylation status and subcellular localisation of NFAT protein. Pharmacological stimuli such as calcium ionophore do not induce activity of an AP-1 driven reporter gene.

A second set of TCR regulated signalling pathways are thought to control activation of AP-1. It is clear that activation of calcium/CN and Ras pathways synergistically induces NFAT/AP-1 activity. Expression of dominant inhibitory forms of either the Ras GTPase or Raf-1, both of which effectively block Ras-dependent signalling pathways, ablated TCR regulation of NFAT/AP-1. Both these signalling molecules also regulate the activity of a reporter gene driven by isolated AP-1 sites. Thus signals derived from Ras are necessary for AP-1 induction. These data were extended in (Genot *et al.*, 1996) who also showed that the activity of another Ras family GTPase, Rac-1, is required for TCR regulation of NFAT/AP-1. Moreover, the data from this study show that Rac-1 acts downstream of Ras to regulate NFAT/AP-1. In T cells there are apparently three Ras effector pathways which are needed for TCR induction of NFAT/AP-1. These are 1) Ras activation of the Raf-1/MEK/ERK cascade, 2) Ras dependent activation of Rac-1 and 2) Ras activation of an as yet uncharacterised third signalling pathway, since 1) and 2) are neccesary but not sufficient for full NFAT/AP-1 induction in this system.

Mast cells do not produce IL-2. They do however produce IL-4 at levels which are thought to be physiologically important for initiation and maintenance of an inflammatory response. It has been recognised from work in T cells that the IL-4 promoter contains multiple composite NFAT/AP-1 sites (Chuvpilo *et al.*, 1993; Rooney *et al.*, 1995), although the regulation of these sites has not been studied in the same detail as for the IL-2 NFAT/AP-1. TCR activation of a reporter gene driven by IL-4 derived NFAT/AP-1 sites is sensitive to Cyclosporin A (Ho *et al.*, 1996), but the role of Ras family GTPases in regulation of this construct has not been explored. There is a limited amount of data upon NFAT/AP-1 sites in the context of the mast cell. FcεR1 activation of RBL2H3 induces a DNA binding complex of NFAT1, Fos and Jun using oligonucleotides derived from the IL-2 promoter (Hutchinson and McCloskey, 1995). Weiss *et al* showed that transcriptional activity of the IL-4 promoter in mast cells requires an NFAT family member (Weiss *et al.*, 1996). Clearly, FcεR1 regulation of IL-4 derived NFAT/AP-1 transcriptional complexes has not been studied in any detail.

6.1.4 Aims.

Potential roles for Ras family GTPases in the regulation of NFAT/AP-1 transcriptional activity have not been examined in the context of the IL-4 promoter in T

or mast cells. However, data from the T cell system suggests that IL-2 derived NFAT/AP-1 sites are critically regulated by Ras and Rac-1. Data presented in Chapter 5 examined the role of Ras and Rac-1 in regulation of the IL-4 promoter. The data showed that amongst the multiple regulatory elements present in the IL-4 (-270) region, there are sites which are targets for Ras and Rac-1. The experiments to be presented in this Chapter examined the following question:

1) Is an NFAT/AP-1 site derived from the IL-4 gene a target for Ras family GTPase signals in the RBL2H3 cell line?

6.2 RESULTS

6.2.1 Inhibition of Protein kinase C signalling does not affect FcεR1 regulation of the NFAT/AP-1 complex.

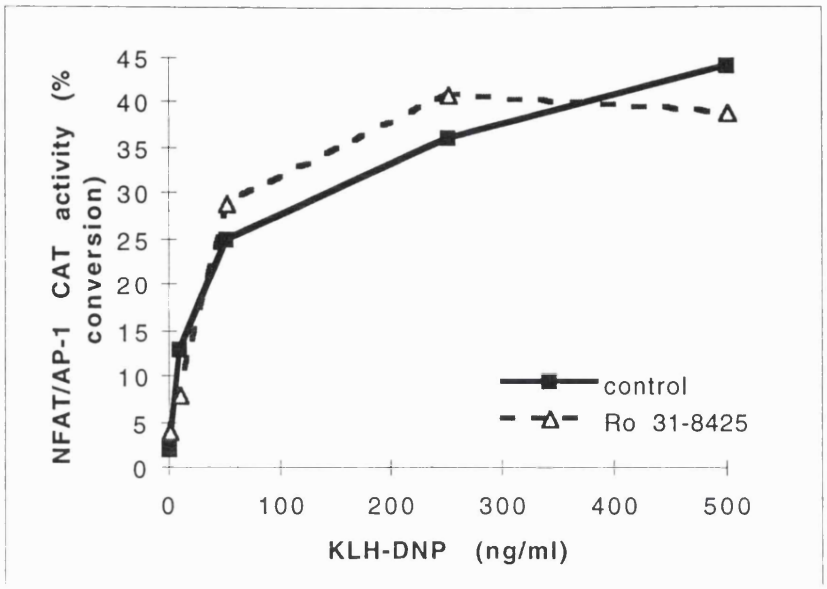
As shown in Chapter 5, Phorbol esters are not able to induce the activity of the NFAT/AP-1 CAT reporter gene derived from the IL-4 promoter. These data suggest that the activation of Protein kinase C is not sufficient to induce NFAT/AP-1 transcriptional activity in RBL2H3. This does not exclude a role for PKC in a necessary but not sufficient contribution to FcεR1 regulation of NFAT/AP-1 complexes. The broad spectrum PKC inhibitor Ro 31-8425 was used in order to examine this point more closely. The data in Figure 6.3a show that application of the Ro-318425 inhibitor did not affect the FcεR1 induction of NFAT/AP-1 CAT activity. In a parallel experiment Ro 31-8425 markedly reduced the PdBu induction of Elk-1 transcriptional activity (Figure 6.3b), suggesting that the efficacy of the inhibitor compound need not be questioned. These data suggest that PKC signals are not important for the FcεR1 induction of NFAT/AP-1 transcriptional activity.

6.2.2 Both Ras and Rac-1 are required for FcεR1 induction of NFAT/AP-1 CAT activity.

Ras and Rac-1 signals regulate the activity of the IL-4 (-270) region in the RBL2H3. Dominant inhibition of either GTPase severely impairs the ability of the FcεR1 to regulate the IL-4 (-270) CAT reporter gene. The NFAT/AP-1 transcriptional complex is one possible target of Ras/Rac-1 signals within this promoter region. In the context of the T cell IL-2 promoter, NFAT/AP-1 complexes are regulated by both Ras and Rac-1. The experiments presented below explore whether the FcεR1 induction of NFAT/AP-1 transcriptional activity requires Ras or Rac-1.

NFAT/AP-1 CAT activity was assayed in RBL2H3 transfected with the dominant inhibitory (N17) Ras mutant. The data in Figure 6.4 show that co-transfection with N17 Ras inhibits FcεR1 induction of NFAT/AP-1 CAT activity. N17 Ras expression decreases FcεR1 induction of this response by 50-60%. This is the maximal inhibition observed, at higher plasmid doses (40μg, data not shown) there is still 40-50% residual activity of the NFAT/AP-1 CAT activity. The effect of N17 Ras is to shift the antigenic sensitivity of NFAT/AP-1 CAT induction by the FcεR1.

a



b

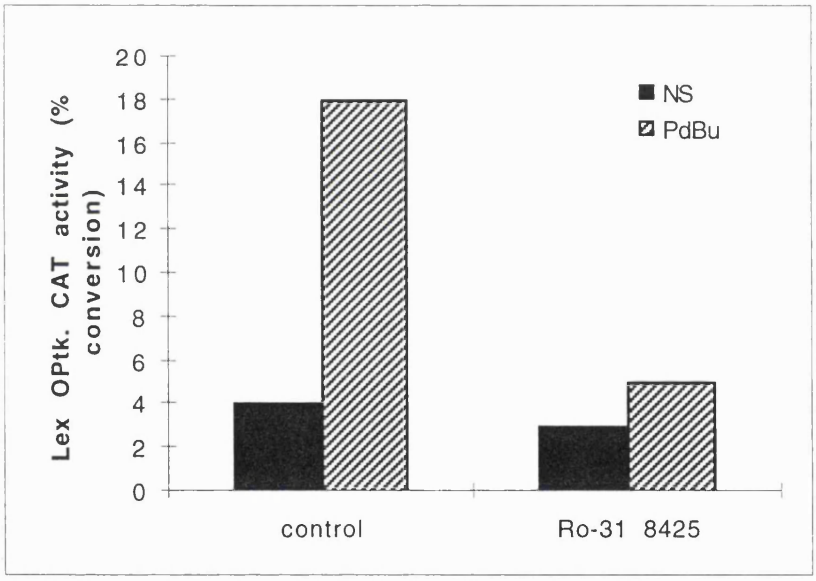


Figure 6.3. Inhibition of Protein kinase C does not affect FcεR1 induction of NFAT/AP-1 CAT activity. a) 10^7 RBL2H3 per point were transfected with 15µg NFAT/AP-1 CAT and recovered for 6h before IgE priming and stimulation with the indicated concentrations of KLH-DNP. Ro 31-8425 was applied 30 min prior to stimulation at a concentration of 250nM, control cells were treated with vehicle alone. b) 10^7 RBL2H3 were transfected with 12µg LexA OPtk.CAT reporter and 6µg LexA Elk-1 C and recovered for 6h before stimulation with 50ng/ml PdBu. Ro 31-8425 was applied as above. Stimulations were for 16h, cells were harvested and CAT activity was assayed as described. Results are representative of 3 experiments.

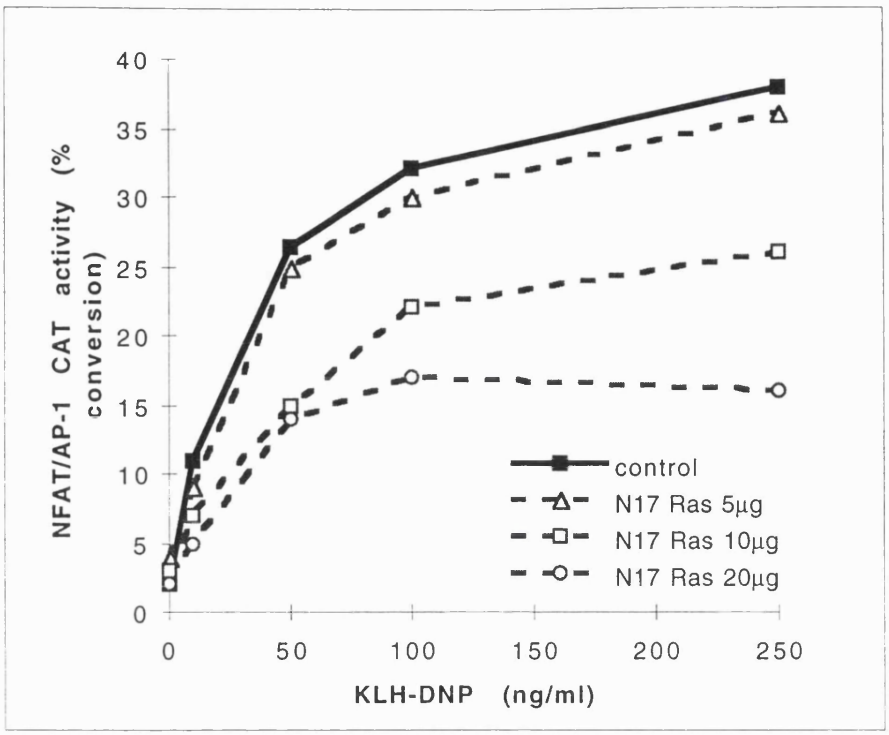


Figure 6.4. Effect of N17 Ras co-transfection on FcεR1 induction of NFAT/AP-1 CAT activity in RBL2H3 10^7 RBL2H3 per point were transfected with 15μg NFAT/AP-1 CAT alone (control) or in combination with the indicated amounts of RSV N17 Ras and recovered for 6h before IgE priming and stimulation with the indicated concentrations of KLH-DNP. Stimulations were for 16h, cells were harvested and CAT activity was assayed as described. Results are representative of 3 experiments.

The experiments represented by Figure 6.5 ask if Rac-1 activity is necessary for FcεR1 activation of NFAT/AP-1. NFAT/AP-1 CAT activity was assayed in RBL2H3 transfected with the dominant inhibitory (N17) Rac-1 mutant. The data show that co-transfection of N17 Rac-1 severely compromises FcεR1 induction of NFAT/AP-1 transcriptional activity. N17 Rac-1 expression inhibits FcεR1 induction of NFAT/AP-1 CAT by 60-70%. At high plasmid doses, N17 Rac-1 effectively removes the ability of the FcεR1 to regulate NFAT/AP-1 transcriptional activity. These data suggest that the activity of the Rac-1 GTPase is absolutely required for FcεR1 induction of NFAT/AP-1 CAT activity.

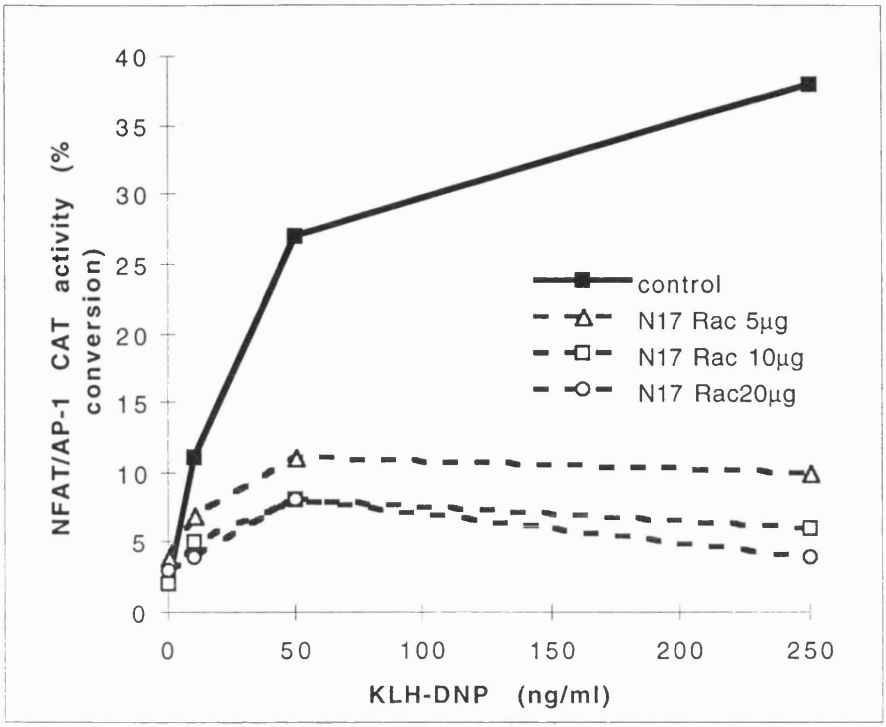


Figure 6.5. Effect of N17 Rac-1 co-transfection on FcεR1 induction of NFAT/AP-1 CAT activity in RBL2H3 10^7 RBL2H3 per point were transfected with 15µg NFAT/AP-1 CAT alone (control) or in combination with the indicated amounts of pEF N17 Rac-1 and recovered for 6h before IgE priming and stimulation with the indicated concentrations of KLH-DNP. Stimulations were for 16h, cells were harvested and CAT activity was assayed as described. Results are representative of 3 experiments.

6.2.3 Effects of activated mutants of Ras , Rac-1 and Rho on NFAT/AP-1 CAT activity.

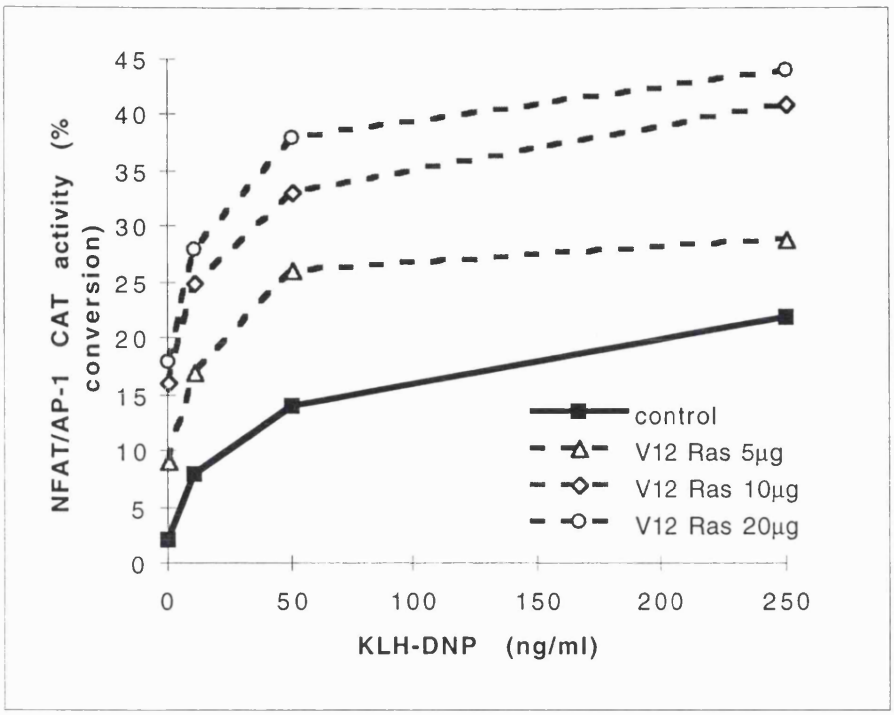
FcεR1 regulation of the NFAT/AP-1 transcriptional complex is shown to require the activity of both Ras and Rac-1. The experiments presented here examine whether dominant active GTPase signals are sufficient to induce NFAT/AP-1 transcriptional activity. FcεR1 induction of NFAT/AP-1 CAT activity was measured in the absence or presence of co-transfected V12 Ras, V12 Rac-1 or the activated mutant of RhoA, V14 Rho.

The data in Figure 6.6a show the effect of dominant active V12 Ras upon FcεR1 induction of NFAT/AP-1 CAT activity. RBL2H3 were transfected with reporter alone or in the presence of increasing doses of pEF V12 Ras plasmid. The data show that in the absence of antigenic crosslinker, V12 Ras co-transfection raises the basal level of NFAT/AP-1 CAT activity. Moreover, V12 Ras strongly potentiates the FcεR1 induction of NFAT/AP-1 CAT, in a manner dose dependent on the amount of co-transfected plasmid. Co-transfection of 10-20µg pEF V12 Ras results in a 3 fold increase in FcεR1 induced NFAT/AP-1 CAT activation. Hence, constitutive activation

of Ras signalling pathways raises basal activity of NFAT/AP-1 CAT and strongly potentiates the effect of FcεR1 stimulation.

Constitutive activation of Rac-1 also results in a strong potentiation of FcεR1 induction of NFAT/AP-1 CAT. The data in Figure 6.6b show the effect of pEF V12 Rac-1 co-transfection upon NFAT/AP-1 CAT activity. In the absence of antigenic crosslinking, V12 Rac-1 also raises basal NFAT/AP-1 CAT activity. Moreover, as for V12 Ras, there is a potentiation of FcεR1 induction of the reporter gene. In the presence of 10-20μg pEF V12 Rac-1, NFAT/AP-1 CAT induction is 3-4 fold greater than in the cells transfected with reporter gene alone. Hence, dominant activation of Rac-1 strongly co-operates with FcεR1 signals to induce NFAT/AP-1 CAT activity.

a



b

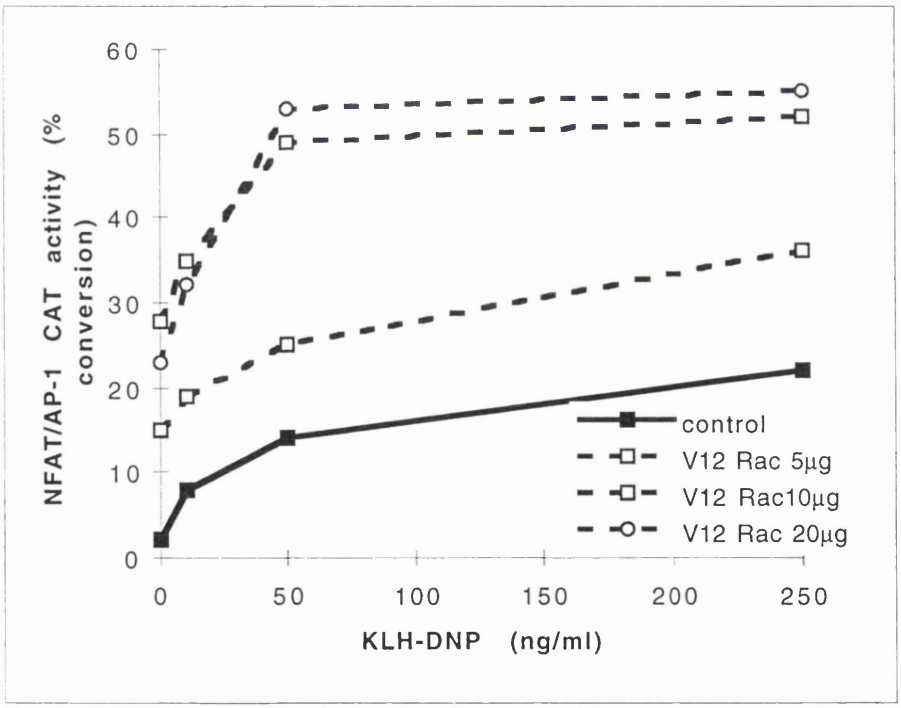


Figure 6.6. V12 Ras and V12 Rac-1 strongly induce NFAT/AP-1 CAT activity in RBL2H3 10^7 RBL2H3 per point were transfected with 15µg NFAT/AP-1 CAT alone (control) or in combination with the indicated amounts of pEF V12 Ras (a) or pEF V12 Rac-1 (b) and recovered for 6h before IgE priming and stimulation with the indicated concentrations of KLH-DNP. Stimulations were for 16h, cells were harvested and CAT activity was assayed as described. Results are representative of 3 experiments.

Consideration of a role for Ras family GTPases in NFAT/AP-1 regulation should not exclude a possible role for Rho. In order to examine this point an activated mutant of RhoA, V14 Rho, was used in co-transfection with NFAT/AP-1 CAT. The data in Figure 6.7 show the effect of co-transfection of pEF V14 Rho upon FcεR1 induction of NFAT/AP-1 CAT activity. It is clear that dominant activation of the Rho GTPase does not potentiate FcεR1 induction of NFAT/AP-1 CAT activity. Co-transfection of 10-20μg pEF V14 Rho resulted in a 20% inhibition of FcεR1 induced NFAT/AP-1 CAT activity. This effect is not profound but was reproduced consistently across a number of experiments.

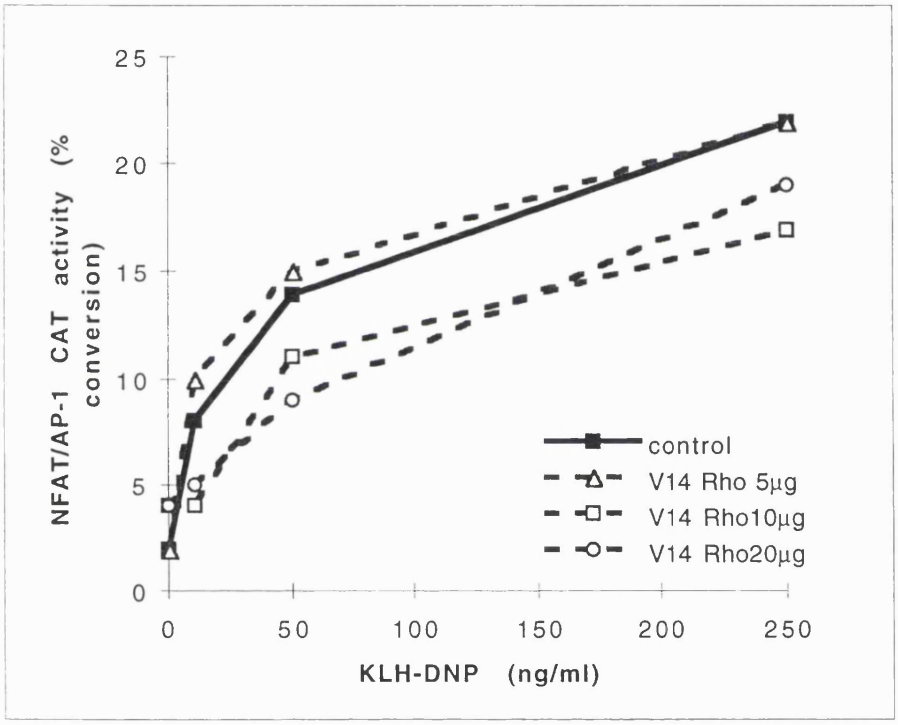


Figure 6.7. The effect of V14 Rho on FcεR1 induction of NFAT/AP-1 CAT activity in RBL2H3 10^7 RBL2H3 per point were transfected with 15μg NFAT/AP-1 CAT alone (control) or in combination with the indicated amounts of pEF V14 Rho and recovered for 6h before IgE priming and stimulation with the indicated concentrations of KLH-DNP. Stimulations were for 16h, cells were harvested and CAT activity was assayed as described. Results are representative of 3 experiments.

The effect of V14 Rho upon NFAT/AP-1 CAT induction by the FcεR1 was unexpected. The data could reflect two situations; 1) that endogenous Rho is in fact a negative regulator of NFAT/AP-1 CAT activity in RBL2H3 or 2) a non-specific effect of dominant active Rho which does not indicate a role for Rho in FcεR1 regulation of NFAT/AP-1. It is well established that activation of Rho in the fibroblast system results in the polymerisation of actin into bundled stress fibres. In the RBL2H3 system

this may form a basis for non-selective inhibition of FcεR1 signalling by V14 Rho. Extensive stress fibre formation may impair FcεR1 signalling by altering the ability of receptors to aggregate and form competent signalling complexes.

A dominant inhibitor of Rho was used to resolve these issues. The *Clostridium botulinum* C3 transferase is a specific potent Rho inactivator, typically inactivating >90% endogenous Rho protein (Aktories, 1994; Aktories and Just, 1995). C3 ADP-ribosylates Rho at a unique site, Asn 41, and inactivates Rho both *in vitro* and *in vivo*. C3 transferase is highly specific for Rho. *In vitro* C3 can ADP-ribosylate purified protein of other Rho family members with low efficiency. However, in a cellular context endogenous GTPases other than Rho are not detectably ADP-ribosylated. Elegant reconstitution experiments by Hill and Treisman further demonstrate C3 specificity (Hill *et al.*, 1995a); C3 effects on SRF-controlled transcription can be reversed only by co-expression of a mutant Rho enzyme which cannot be ribosylated. The effect of C3 is not overcome by wild type Rho or by co-expression of other Rho family members.

If Rho is in fact a negative regulator of NFAT/AP-1 activity in RBL2H3, then dominant inhibition of Rho using C3 transferase would be expected to enhance the FcεR1 induction of NFAT/AP-1 CAT activity. The data in Figure 6.8 show the effect of co-transfection of pEF C3 transferase upon FcεR1 induction of NFAT/AP-1 CAT activity. The data show that C3 transferase does not significantly inhibit FcεR1 induction of NFAT/AP-1 CAT activity. Taken together, the lack of potentiation by V14 Rho and lack of inhibition by C3 suggest that Rho signals do not significantly contribute to FcεR1 induction of NFAT/AP-1 CAT activity.

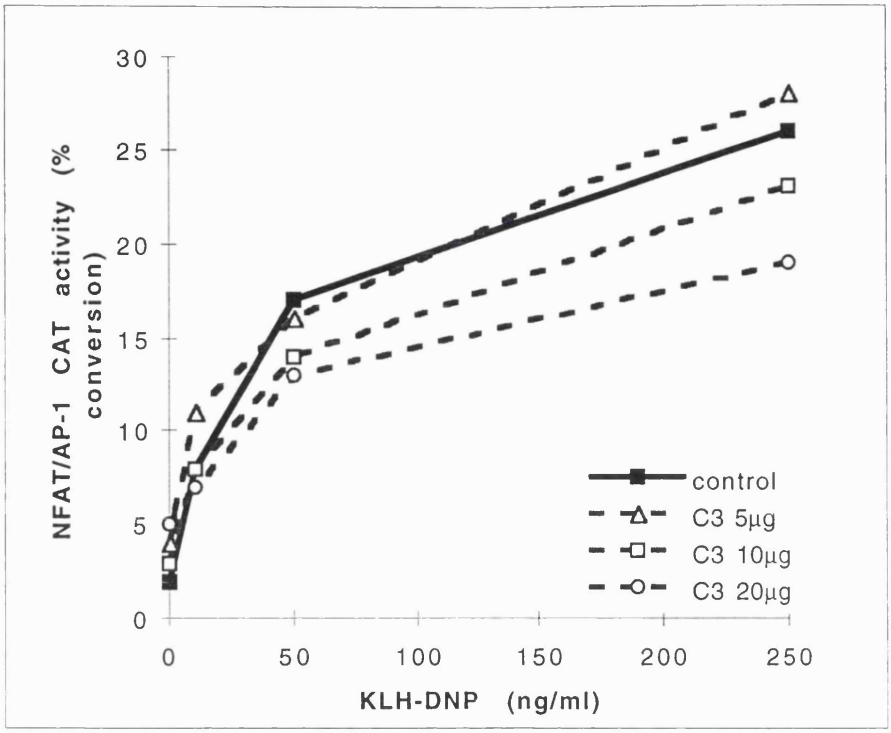


Figure 6.8. Effect of C3 transferase co-transfection on FcεR1 induction of NFAT/AP-1 CAT activity in RBL2H3 10^7 RBL2H3 per point were transfected with 15µg NFAT/AP-1 CAT alone (control) or in combination with the indicated amounts of pEF C3 transferase and recovered for 6h before IgE priming and stimulation with the indicated concentrations of KLH-DNP. Stimulations were for 16h, cells were harvested and CAT activity was assayed as described. Results are representative of 3 experiments.

6.2.4 Ras regulation of NFAT/AP-1 CAT activity does not require the Raf-1/MEK/ERK effector pathway.

The data presented in section 6.2.3 suggest that the activity of the Ras GTPase is necessary but not sufficient for induction of NFAT/AP-1 CAT activity. As described for the regulation of Elk-1 in Chapter 4, a role for Ras raises the issue of the relevant Ras effector pathway for that response. In the case of Elk-1 regulation, this was found to be the 'classical' Raf-1/MEK/ERK signalling cascade. The experiments presented below ask if this is also the effector mechanism relevant to FcεR1 induction of NFAT/AP-1 transcriptional activity.

Two reagents are available for examination of a possible role for the Raf-1/MEK/ERK signalling cascade. The Raf-1 CAAX fusion protein is a membrane targeted version of the Raf-1 serine/threonine kinase. Membrane localisation of Raf-1 renders the enzyme constitutively active and results in activation of MEK and consequently ERK. In the case of Elk-1 activation, a Raf-1/MEK/ERK regulated event, Raf-1 CAAX and V12 Ras are equipotent. This reflects that both Ras and Raf-1 can drive Elk-1 activation by the Raf-1/MEK/ERK cascade. The effect of Raf-1 CAAX

upon NFAT/AP-1 CAT activation was tested in the experiment shown in Figure 6.9. The data show that as described above, V12 Ras strongly potentiates FcεR1 induction of NFAT/AP-1 CAT activity. The activated Raf-1 CAAX mutant does not mimic V12 Ras; hence constitutive activation of the Raf-1/MEK/ERK cascade is not sufficient to drive induction of NFAT/AP-1 CAT.

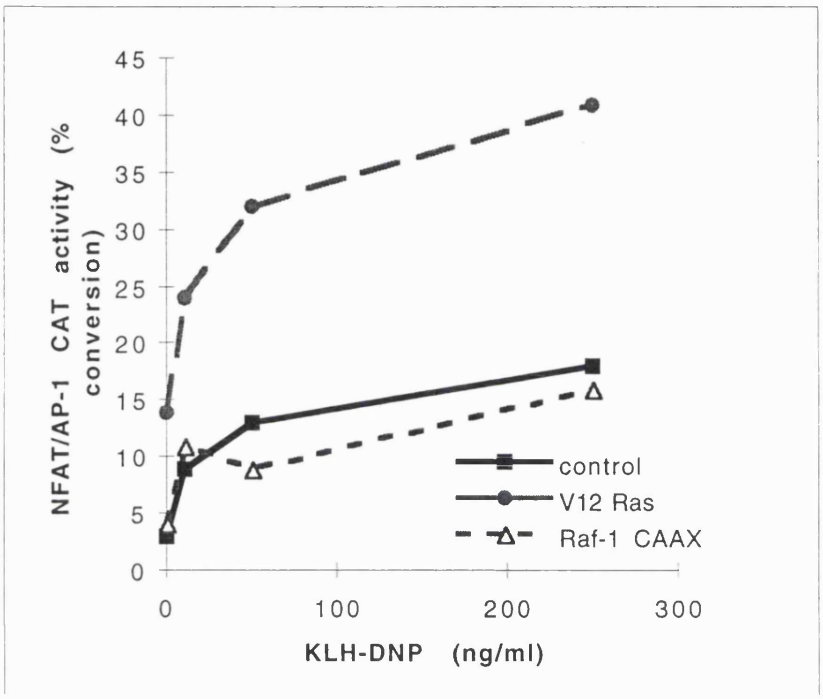


Figure 6.9. Effect of V12 Ras and Raf-1 CAAX upon FcεR1 induction of NFAT/AP-1 CAT activity in RBL2H3 10^7 RBL2H3 per point were transfected with 15μg NFAT/AP-1 CAT alone (control) or in combination with either 15μg pEF V12 Ras or 15μg pEF Raf-1 CAAX and recovered for 6h before IgE priming and stimulation with the indicated concentrations of KLH-DNP. Stimulations were for 16h, cells were harvested and CAT activity was assayed as described. Results are representative of 3 experiments.

The second reagent available for study of the Raf-1/MEK/ERK cascade is the MEK inhibitor compound PD 098059. Application of PD 098059 ablates FcεR1 induction of Elk-1 transcriptional activity, indicating that this is a MEK/ERK dependent event. The experiment in Figure 6.10 addresses the effect of PD 098059 upon FcεR1 induction of NFAT/AP-1 CAT. As a control for the efficacy of this reagent, the effect of PD 098059 on FcεR1 induction of Elk-1 transcriptional activity was measured in parallel. The data show that whilst PD 098059 markedly inhibits FcεR1 induction of Elk-1 (LexA OPtk CAT activity), this reagent does not affect FcεR1 stimulation of NFAT/AP-1 complexes.

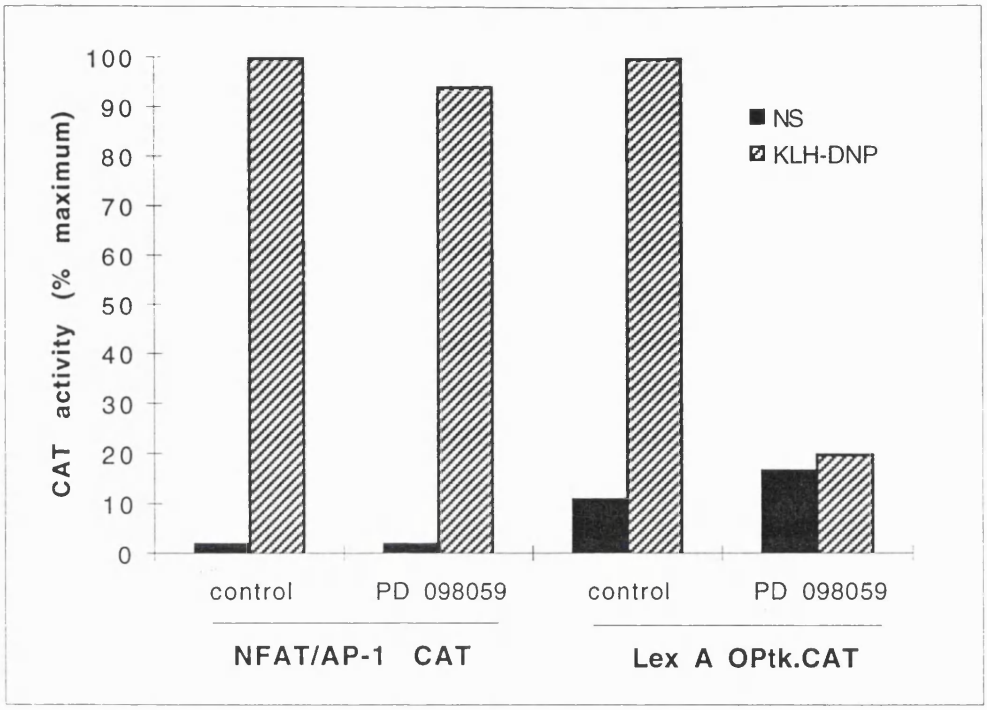


Figure 6.10. Effect of PD 098059 upon FcεR1 induction of NFAT/AP-1 and Elk-1 transcriptional activity in RBL2H3. 10^7 RBL2H3 per point were transfected with 15μg NFAT/AP-1 CAT alone or 12μg pEF Lex AOptkCAT with 6μg pEF Lex A Elk-1C. Cells were recovered for 6h before IgE priming and stimulation with the indicated concentrations of KLH-DNP. PD 098059 (25μM) was applied 30 min prior to stimulation. Stimulations were for 16h, cells were harvested and CAT activity was assayed as described. Results are representative of 3 experiments.

Two pieces of data shown above suggest that the FcεR1 induction of NFAT/AP-1 transcriptional activity is not dependent upon activity of the Raf-1/MEK/ERK cascade. Constitutive activation of the cascade using Raf-1 CAAX is not sufficient to induce NFAT/AP-1 CAT activity. Moreover, inhibition of the central kinase of the cascade, MEK, does not affect FcεR1 induction of NFAT/AP-1. Hence the Raf-1/MEK/ERK cascade is not the Ras effector pathway relevant to FcεR1 induction of NFAT/AP-1 activity. The remaining candidate Ras effector pathways are uncharacterised in RBL2H3 and are likely to be numerous. Data from other cell systems suggests that Ras effectors may include the Rac-1 GTPase, PI 3-kinase and a GDS protein for the Ral GTPase. Data to be presented in Chapter 7 will address this issue further.

6.2.5 Effector loop mutants of the Rac-1 GTPase selectively activate a subset of Rac-1 responses.

The data in Figure 6.5 showed that the FcεR1 induction of NFAT/AP-1 CAT activity absolutely requires activity of the Rac-1 GTPase. In various cell systems

considerable effort has been made to identify Rac-1 effector molecules. By definition, the transducers of Rac-1 signals play important roles in processes such as rearrangements of the actin cytoskeleton and oncogenic transformation. Currently identified direct Rac-1 effectors in mammalian cells (reviewed in (Reif and Cantrell, 1997)) include the p21 associated kinases (PAKs), the phagocyte unique NADPH oxidase (Phox), and the p160 ROCK serine/threonine kinase. Activity of the Jun N-terminal kinase (JNK) is also functionally regulated by Rac-1. The p160 ROCK kinase was first identifed as a Rho binding protein, whilst PAK and JNK activation are apparently shared between CDC42Hs and Rac-1, but not Rho.

Effector loop mutants of Rac-1 have been identified which selectively activate certain Rac-1 target molecules. This mutational strategy was used by the laboratory of Alan Hall to generate expression vectors bearing Rac-1 mutants altered in two ways from wild-type: 1) The L61 mutation renders the GTPase insensitive to GAP activity, similarly to V12 mutation described previously. 2) A series of effector loop mutations were generated which cannot interact with a subset of Rac-1 effectors (Dieckmann *et al.*, 1995). Lamarche *et al* characterised and functionally assessed these mutants in a fibroblast cell line (Lamarche *et al.*, 1997). Their data are summarised below (Figure 6.11).

mutant	effector molecule interaction			functional responses
	PAK	ROCK	Phox	
Rac-1 L61	+	+	+	MR, FC, JNK
L61 Y40C		+	+	MR, FC
L61 F37A	+		+	FC, JNK

Figure 6.11. Interactions of Rac-1 mutants and the functional responses which they can induce in fibroblasts. Effector loop mutants in Rac-1 activate a subset of Rac-1 dependent processes. PAK, p21 associated kinase; ROCK, Rho associated coiled-coil containing kinase; Phox, phagocyte oxidase; MR, membrane ruffles; FC, focal complex formation; JNK, Jun N-terminal kinase activation. From (Lamarche *et al.*, 1997).

Expression vectors bearing effector mutants of Rac-1 were generously made available by Dr Alan Hall, LMCB, London. These mutants were used in transient transfection of RBL2H3. FcεR1 induction of NFAT/AP-1 CAT activity was assayed in the presence of these mutants. The data in Figure 6.12 show the results of Rac-1

L61, L61 Y40C and L61 F37A co-transfection upon NFAT/AP-1 CAT activity in comparison with the effect of V12 and N17 Rac-1. The GAP resistant V12 and L61 mutants of Rac-1 both strongly induce NFAT/AP-1 CAT activity in the absence of other stimuli, to a level of CAT activity similar to that in control cells stimulated via the FcεR1. Moreover, these mutants potentiate the FcεR1 induction of NFAT/AP-1 CAT.

The data in Figure 6.12 also explore the effects of the L61 Y40C and L61 F37A amino-acid substitutions upon NFAT/AP-1 CAT activity. The effect of L61 Y40C is comparable to that of the V12 and and L61 single substitution mutants. In contrast, co-transfection with the L61 F37A mutant blocks FcεR1 induction of NFAT/AP-1 CAT activity. This effect is comparable to the inhibition observed with the dominant inhibitory Rac-1 mutant N17 Rac-1.

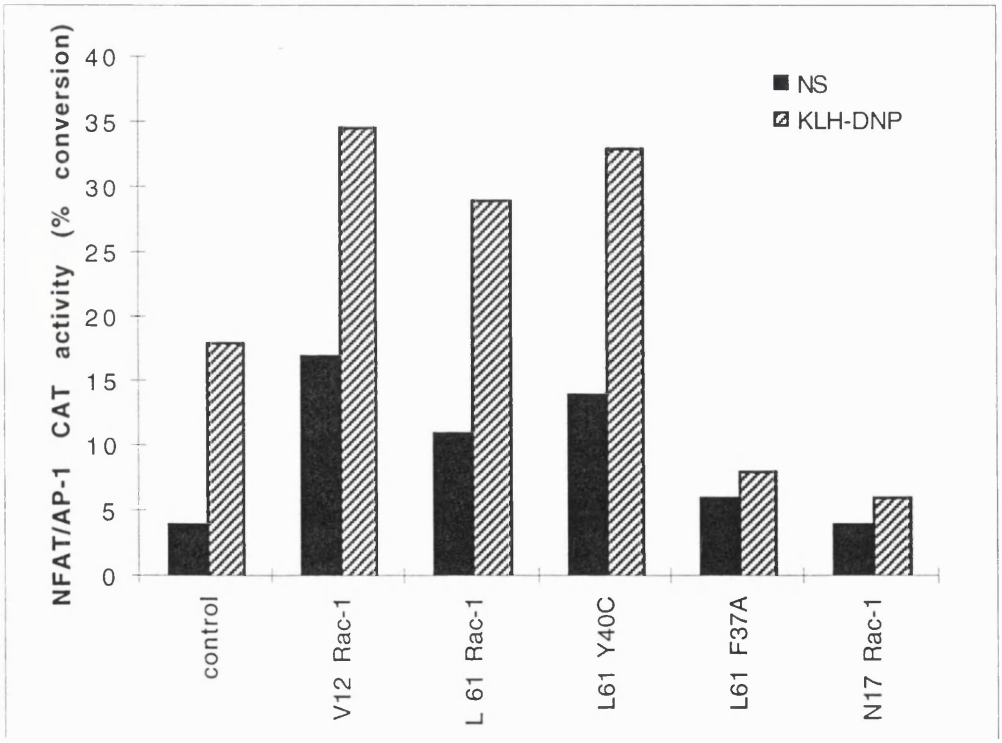


Figure 6.12. Effect of Rac-1 mutants upon FcεR1 induction of NFAT/AP-1 CAT activity in RBL2H3 10^7 RBL2H3 per point were transfected with 15μg NFAT/AP-1 CAT alone (control) or in combination with 20μg pEF V12 Rac, 20μg pRK L61 Rac-1, 20μg pRK L61Y40C Rac-1 , 20μg pRK L61F37A Rac-1 or 20μg pEF N17 Rac-1 and recovered for 6h before IgE priming and stimulation with the indicated concentrations of KLH-DNP. Stimulations were for 16h, cells were harvested and CAT activity was assayed as described. Results are representative of 3 experiments.

In parallel with the NFAT/AP-1 CAT activity assays represented above, the expression of the mutant Rac-1 proteins was assessed by Western blot. These mutants bear a *myc* epitope tag enabling recognition of transfected as opposed to endogenous Rac-1 protein. The 9E10 monoclonal antibody is specific for the *myc* epitope (Evan *et*

al., 1985). In these experiments RBL2H3 were transfected with equivalent amounts of the relevant plasmid to those used in the NFAT/AP-1 CAT assay. Cell lysates were prepared and proteins were resolved by SDS-PAGE. The data in Figure 6.13 show an anti-*myc* Western analysis of lysate from untransfected cells or RBL2H3 transfected with V12 Rac-1, L61 Rac-1, L61 Y40C or the L61 F37A mutant. V12 and L61 singly substituted mutants were both easily detected. Similarly, the L61 Y40C mutant could be detected by 9E10 Western blotting. However, in repeated experiments, 9E10 could not detect the expression of the L61 F37A mutant.

These data suggest that either 1) the L61 F37A Rac-1 mutant is expressed at a level below the sensitivity of the 9E10 Western analysis or 2) that the *myc* epitope is for some reason not available in the L61 F37A Rac-1 mutant. This lack of expression data for the L61 F37A mutant means that the significance of the inhibition of FcεR1 induction of NFAT/AP-1 CAT activity observed with L61 F37A could not be assessed.

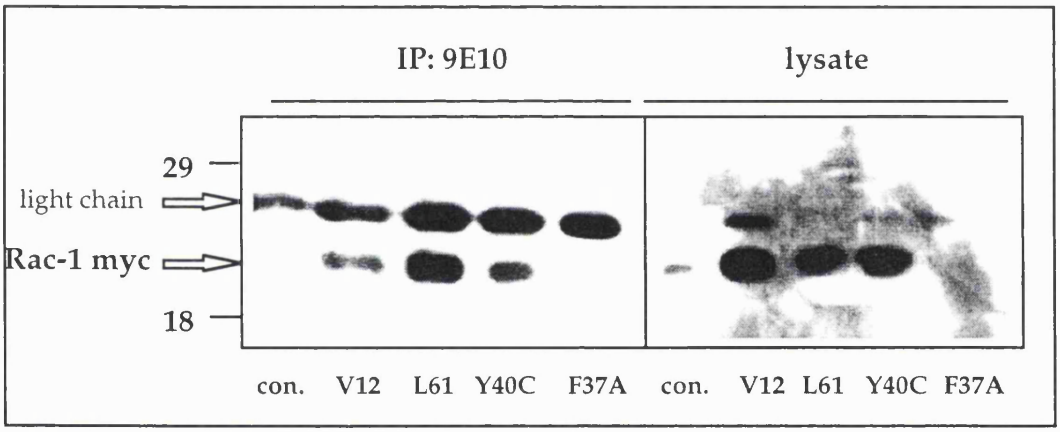


Figure 6.13. 9E10 (anti-myc) Western analysis of expression of Rac-1 mutant proteins in RBL2H3 10^7 RBL2H3 per point were transfected with 20μg pEF vector (con), 20μg pEF V12 Rac, 20μg pRK L61 Rac-1, 20μg pRK L61Y40C Rac-1 or 20μg pRK L61F37A Rac-1 and recovered for 6h. Cells were harvested and lysed prior to immunoprecipitation as described in Materials and Methods. Post nuclear lysate from $5x10^6$ cell equivalents was prepared and acetone precipitated as described. For 9E10 immunoprecipitation (IP), a single preclear step was performed using insoluble Protein A. Myc-tagged proteins from 10^7 cell equivalents were immunoprecipitated using 5μg/point 9E10 coupled to Protein A sepharose. Proteins were resolved on 17% SDS-PAGE, and transferred to PVDF membrane. Western analysis was performed with with 1μg/ml 9E10.

6.3 DISCUSSION.

NFAT/AP-1 transcriptional complexes regulate a variety of immune response gene targets, including the gene for IL-4. FcεR1 regulation of the IL-4 promoter region involves a number of signalling pathways. Using dominant negative signalling proteins and small molecule inhibitors a number of signals were described as important for FcεR1 regulation of IL-4 gene induction. These included calcium/calcineurin activation and signals derived from the Ras and Rac-1 GTPases. The data presented in Chapter 6 addressed whether the NFAT/AP-1 transcriptional complex is a target for any of these signalling pathways in the RBL2H3 system.

6.3.1 The Ras and Rac-1 GTPases, but not Protein kinase C, regulate NFAT/AP-1 CAT activity in RBL2H3.

Inhibition of Protein kinase C using the Ro 31-8425 inhibitor does not affect the ability of the FcεR1 to regulate NFAT/AP-1 transcriptional complexes. These data suggest that NFAT/AP-1 is not a target in the IL-4 promoter for PKC mediated signals. In contrast, dominant inhibition of either Ras or Rac-1 resulted in compromised FcεR1 regulation of NFAT/AP-1 induction. These effects were selective for Ras and Rac-1; inactivation of the Rho GTPase using the C3 ADP-ribosyltransferase did not affect FcεR1 induction of NFAT/AP-1. In the T cell system, the signalling requirements for activation of NFAT/AP-1 complexes in the context of the IL-2 promoter have been examined. In addition to calcium/CN regulation of NFAT nuclear import, three T cell receptor induced Ras signalling pathways are required for IL-2 NFAT/AP-1 induction. These Ras effector pathways are 1) the Raf-1/MEK/ERK cascade, 2) a Ras dependent Rac-1 signalling pathway and 3) an uncharacterised non-MEK Ras effector pathway (Genot *et al.*, 1996).

Hence, there are marked similarities between the signalling requirements for FcεR1 regulation of IL-4 NFAT/AP-1 and TCR induction of IL-2 derived NFAT/AP-1 complexes. In both systems the activity of Ras and Rac-1 is required in concert with calcium/CN signals. A significant difference between the two systems is in the requirement for the Raf-1/MEK/ERK signalling cascade. TCR regulation of the IL-2 NFAT/AP-1 site requires the activity of this pathway. In contrast, inhibition of the central MEK kinase does not affect FcεR1 induction of the IL-4 NFAT/AP-1 complex. These data also highlight an important difference between FcεR1 regulation of two independent nuclear targets. The FcεR1 uses Ras to regulate both Elk-1 and NFAT/AP-1 complexes. Activity of the Raf-1/MEK/ERK cascade is necessary and sufficient for FcεR1 induction of Elk-1 transcriptional activity. FcεR1 regulation of NFAT/AP-1 is independent of Raf-1/MEK/ERK activity. Hence, in RBL2H3, FcεR1 derived Ras signals regulate disparate nuclear targets via distinct effector pathways.

This reflects one of the key aspects of GTPase signalling; the efficiency achieved where a single upstream input may result in regulation of multiple target molecules.

6.3.2 Ras activity is necessary for FcεR1 induction of NFAT/AP-1 transcriptional activity.

Dominant inhibition of the Ras GTPase impairs FcεR1 induction of NFAT/AP-1 CAT activity. This effect is not a complete inhibition of NFAT/AP-1 induction. At high doses of inhibitory Ras, where Ras dependent processes such as Elk-1 induction are ablated, there is significant residual NFAT/AP-1 CAT activation. Hence Ras activity is necessary but not sufficient for FcεR1 induction of NFAT/AP-1 transcriptional activity in RBL2H3. The effect of N17 Ras is to shift the antigen sensitivity of FcεR1 induced NFAT/AP-1 CAT activity. Ras is not solely activated by antigen receptor triggering in mast cells, but can be activated by cytokines such as IL-3 (Wodnar Filipowicz and Moroni, 1990; Satoh *et al.*, 1991) and GM-CSF (Satoh *et al.*, 1991). Activation of Ras stimulatory pathways by integrins, which are likely to be involved in mast cell adherence to a tissue substratum, has also been documented (Schlaepfer *et al.*, 1994). It is reasonable to expect that during the early course of an inflammatory response, mast cells would be exposed to Ras activating cytokines such as IL-3 and GM-CSF produced by antigen-activated T cells. The effect of Ras activation upon IL-4 NFAT activity is essentially a shifting in receptor sensitivity. Hence, exposure of mast cells to Ras activating cytokines may be viewed as an important priming step which alters the mast cell responsiveness to a given antigen dose. Ras activation by contextual signals may in fact set a threshold for subsequent antigenic induction of events such as transcriptional activation.

Across a number of cell systems various Ras effector molecules have been characterised (Marshall, 1996). These include Raf-1, PI 3-kinase, a GDS for the Ral GTPase and Rac-1. In the context of FcεR1 induction of NFAT/AP-1 activity the Raf-1/MEK/ERK signalling cascade is not the relevant Ras effector pathway. Identification of the Ras effector linking the FcεR1 and NFAT/AP-1 activity is not a trivial matter and has not been addressed by experiment. In the mast cell system no Ras effectors other than Raf-1 have been clearly identified, although FcεR1 crosslinking is known to result in PI 3-kinase activation. It is entirely possible that there are novel Ras effectors to be characterised in this and other cell systems. Future identification of Ras effectors in mast cells could be exploited to identify the relevant pathways for FcεR1 induction of NFAT/AP-1. The strategy of using effector loop mutants of GTPases which are defective in signalling to a subset of effector molecules is established for both Ras and Rac-1. These type of mutants would allow an assessment of whether FcεR1 induction of NFAT/AP-1 is dependent upon a particular Ras effector molecule.

It is recognised that certain cellular responses to Ras require the activity of other Ras family GTPases; notably Ras mediated transformation of fibroblasts requires signalling pathways mediated by Rac-1 and RhoA. Moreover, Rac-1 couples Ras to the signalling mechanism which control rearrangements of the actin cytoskeleton in fibroblasts. The requirement for both Ras and Rac-1 function for FcεR1 induction of NFAT/AP-1 complexes is consistent with two models. 1) Rac-1 may act as a downstream effector of Ras or 2) Rac-1 and Ras are components of distinct parallel signalling pathways which converge on NFAT/AP-1 to fully activate the transcriptional complex. These possibilities are difficult to resolve and may not be mutually exclusive. Data to be presented in Chapter 7 examine this issue using activated GTPases to rescue the dominant inhibition of NFAT/AP-1 induction.

6.3.3 Rac-1 activity is absolutely required for FcεR1 induction of NFAT/AP-1 CAT activity.

The NFAT/AP-1 complex is identified as a target for Rac-1 signalling pathways in RBL2H3. Expression of active Rac-1 dramatically enhances FcεR1 induction of NFAT/AP-1 transcriptional activity, whereas dominant inhibition of Rac-1 severely abrogates the NFAT/AP-1 response to FcεR1 stimulation. In contrast with the data obtained using dominant inhibitory Ras, in the presence of N17 Rac-1 there is no residual FcεR1-induced NFAT/AP-1 CAT activity. A blockade in Rac-1 signalling effectively removes the ability of the FcεR1 to regulate NFAT/AP-1 complexes.

In the T cell system a requirement for Rac-1 in regulation of NFAT/AP-1 complexes has been ascribed to Rac-1 induction of AP-1 dimers. This model is largely based upon the ability of Rac-1 to regulate the transcriptional activity of isolated AP-1 complexes. Hence V12 Rac-1 strongly induces the activity of a CAT reporter gene driven by a trimerised AP-1 site. It is not possible to perform a similar experiment in the RBL2H3 system, since AP-1 dimers do not transactivate independently of NFAT in these cells. Therefore, while it seems likely that Rac-1 signals target AP-1 in mast cells as in T cells, this issue is not fully resolved. The induction of NFAT/AP-1 CAT activity by V12 Rac-1 in RBL2H3 is sensitive to the action of Cyclosporin A, an inhibitor of Calcineurin activity (Turner and Cantrell, 1997). This suggests that the activatory effects of Rac-1 are subject to a requirement for CN activity (ie. for NFAT nuclear import). Hence, FcεR1 induction of NFAT/AP-1 transcriptional activity requires at least the convergent action of Rac-1 and CN.

Rac-1 plays a pivotal role in FcεR1 regulation of NFAT/AP-1 complexes. These data raise the issue of the identity of the Rac-1 effector pathway involved in this response. Across a number of cell systems a number of Rac-1 effector molecules have been identified. Proteins that can bind directly to GTP bound Rac-1 include members of the PAK serine/threonine kinase family, the ribosomal p70S6 kinase, the ROCK

kinase and the Phagocyte oxidase molecule Phox. Indirectly, Rac-1 has also been shown to activate two MAP kinase family members; JNK and the p38(RK) kinase. At the present time it is not possible to definitively ascribe a role in FcεR1 regulation of NFAT/AP-1 to a particular effector pathway. However, it is possible to exclude ceratin candidate signals from a contribution to this response.

The immunosuppressant compound Rapamycin is an inhibitor of the p70 S6 kinase molecule. It is established that IL-2 production in T cells is not sensitive to Rapamycin (Dumont *et al.*, 1990). Moreover, IL-4 mRNA production is not inhibited by Rapamycin in T cells. There is a small molecule inhibitor of the p38 (RK) kinase, SB 203580 (Cuenda *et al.*, 1995). Preliminary data show that this compound does not modulate NFAT responses to FcεR1 activation. Hence it is unlikely that either of these enzymes are responsible for transduction of the Rac-1 signal to NFAT/AP-1. There is no small molecule inhibitor of the JNK kinase analogous to the PD 098059 and SB 203580 compounds available. Such a reagent would be a convenient tool to probe a putative role for JNK in coupling the FcεR1 to NFAT/AP-1. JNK may be seriously considered in this context since a number of links between the FcεR1, Rac-1and JNK have been found; 1) The FcεR1 regulates kinase activity of JNK (Ishizuka *et al.*, 1996), 2) The FcεR1 regulates Vav-1 (Margolis *et al.*, 1992). Vav-1 is an exchange factor for Rac-1 (Crespo *et al.*, 1997) and Rac-1 regulation of JNK has been demonstrated to be potentiated by overexpression of Vav-1 in a Cos cell/FcεR1 system (Teramoto *et al.*, 1997).

Experiments were attempted using effector loop mutants of Rac-1 to examine the roles of particular Rac-1 effectors in FcεR1 induction of NFAT/AP-1 CAT. The preliminary data from these experiments is shown in Chapter 6. The expression of the V12, L61 and L61 Y40C mutants of Rac-1 could be confirmed by Western blot. The expression of L61 F7A could not be confirmed by Western blot and hence the effect of this mutant on NFAT/AP-1 CAT activity could not be assessed. The activated L61 Rac-1 mutant strongly induced NFAT/AP-1 CAT activity. The L61 Y40C mutant induces NFAT/AP-1 CAT activity similarly to the Rac-1 L61 singly substituted protein. Lamarche *et al* characterised the protein-protein interactions and activatory capabilities of the Rac-1 effector loop mutants in a fibroblast cell system (Lamarche *et al.*, 1997). Their data show that the L61 Y40C mutant can interact with the ROCK Rac-1 effector kinase and can drive Rac-1 regulated rearrangements in the actin cytoskeleton to generate membrane ruffles and focal complex formation. The L61 Y40C mutant is unable to interact with the PAK kinase and does not induce kinase activity of either PAK or JNK. If these data are extrapolated to the RBL2H3 system, then it would seem that a Rac-1 mutant (L61 Y40C) which cannot activate JNK or PAK is able to activate NFAT/AP-1 to a level similar to that observed with the GAP-resistant V12 Rac-1.

There are a number of qualifications to be noted at this point. First, the interactions of these effector loop mutants are not characterised in the RBL2H3 system. Second, the interactions of these mutants examined in the fibroblast do not extend to the full range of Rac-1 effectors characterised in that system. For example, the cytoskeletal regulatory protein POR-1 (Van Aelst *et al.*, 1996) and a lipid phosphatidylinositol 5-kinase activity (Hartwig *et al.*, 1995) are also Rac-1 targets in fibroblasts. There is no data available on whether the mutants used here may be compromised in its ability to regulate any of these novel Rac-1 effectors. It is clearly important not to over-interpret the data obtained with the L61 Y40C mutant, but this is an interesting preliminary observation.

FcεR1 regulation of the Ras GTPase is a pivotal control point which apparently links the FcεR1 to disparate nuclear targets. Ras regulates Elk-1, a transcription factor involved in immediate early gene induction, via the 'classical' Raf-1/MEK/ERK kinase cascade. In the mast cell system Ras also regulates NFAT/AP-1 complexes and hence the promoters of immunoregulatory genes such as that for IL-4 . In this case however, a Ras effector pathway distinct from Raf-1/MEK/ERK is involved. NFAT/AP-1 induction in RBL2H3 is critically dependent upon activity of the Rac-1 GTPase. FcεR1 regulation of Rac-1, possibly via activation of the Vav guanine nucleotide exchange factor, may act in parallel or series with Ras signals to regulate NFAT/AP-1 complexes. These observations are summarised in Figure 6.14.

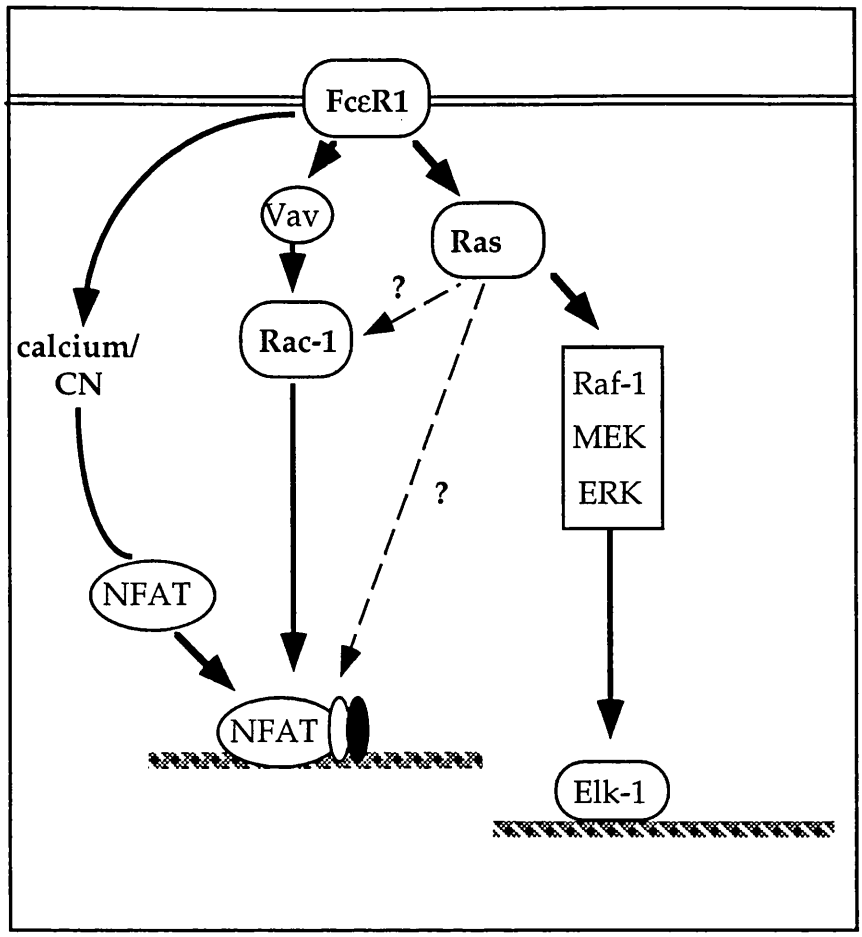


Figure 6.14. Mechanisms for FcεR1 regulation of transcription factor targets in RBL2H3. Ras signals through the Raf-1/MEK pathway are necessary and sufficient for Elk-1 activation but not required for FcεR1 induction of NFAT/AP-1. The NFAT/AP-1 complex integrates multiple FcεR1 derived signals. Calcium regulates NFAT subcellular localisation, while the activity of Ras and Rac-1 is required either in parallel or series.

The FcεR1 absolutely requires Rac-1 for activation of NFAT/AP-1. This is an intriguing observation since it suggests that Rac-1 controls a critical step in the induction processes leading to transcriptional activation of the NFAT/AP-1 complex. By analogy with the T cell system, both Ras and Rac-1 signals might be expected to target the AP-1 dimer. This component is crucial for transcriptional activation of NFAT/AP-1 complex in both systems. However, were both Ras and Rac-1 targeting the AP-1 dimer in the RBL2H3, possibly in a linear pathway, it would be predicted that both would be required by the FcεR1 to the same degree. In fact, the FcεR1 requirement for Ras and Rac-1 in NFAT/AP-1 induction is quite different. These data provide the basis for a hypothesis that Ras and Rac-1 are not targeting the same aspect of NFAT/AP-1 induction. The data to be presented in Chapter 7 will address this issue.

CHAPTER SEVEN

FcεR1 regulation of NFAT sub cellular localisation in RBL2H3 involves the Ras family GTPase Rac-1.

7.1 INTRODUCTION

NFAT family members regulate numerous gene targets coding for immuno-modulatory proteins. NFAT proteins bind DNA in the context of a co-operative interaction with an AP-1 dimer. The regulation of NFAT/AP-1 transcriptional activity in FcεR1 stimulated RBL2H3 was the focus of the work presented in Chapter 6. In addition to calcium signals, the Ras family GTPases Ras and Rac-1 were found to regulate an NFAT/AP-1 reporter gene derived from the IL-4 promoter. The data showed that Ras activity is necessary but not sufficient for FcεR1 induction of NFAT/AP-1 transcriptional activity. Inhibition of Ras activity results in a shift in the antigen receptor dose response for FcεR1 activation. Rac-1 is absolutely required for NFAT/AP-1 activation in RBL2H3. The work presented in this Chapter was directed towards identifying the basis for this unequivocal requirement for Rac-1 signals.

Conventional models for NFAT/AP-1 activation suggest that the basis for the GTPase requirement discussed above lies in the regulation of the AP-1 dimer. However, the difference in significance of the two GTPases implicated and the absolute nature of the Rac-1 requirement suggests that in the FcεR1 system this model may not be valid. Therefore, the role of Ras and Rac-1 GTPases in non-AP-1 aspects of NFAT regulation was studied. The nuclear import of NFAT protein and hence its access to DNA is a highly regulated commitment step in the activation pathways for NFAT/AP-1 regulated promoters. The work presented here details studies using a Green Fluorescent Protein (GFP) reporter gene assay for the sub cellular localisation of NFATC1 protein in the mast cell line RBL2H3. Activated and dominant inhibitory GTPase mutants were used to elucidate the signalling requirements for NFATC1 translocation from the cytosol to the nucleus under the control of the FcεR1.

The data presented here show that nuclear import of NFATC1 in response to antigenic stimulation of mast cells via the FcεR1 absolutely requires Rac-1, but not the Ras GTPase. This is a selective effect of Rac-1 which is independent of published links between Rac-1 and the actin cytoskeleton. Rac-1 does not target the general nuclear import machinery, rather, Rac-1 signals affect NFAT phosphorylation status and hence commitment to nuclear import. GTPase derived signals should therefore not be considered solely in the context of AP-1 dimer regulation by antigen receptors.

These data reflect a novel role for Rac-1 as a key player in the control of NFAT sub cellular localisation.

7.1.2 NFAT activation is a multi-stage process.

Extensive studies have been performed examining the events which comprise NFAT activation. A consensus model has been drawn for the regulation of transcriptional activity of NFAT/AP-1 complexes. showing a requirement for antigen receptor to regulate 1) AP-1 proteins via Ras family GTPases and 2) a calcium/Calcineurin pathway which regulates the sub cellular localisation of NFAT protein (Rao *et al.*, 1997). The latter pathway is the focus of much analysis because of its therapeutic importance as the target of the macrolide immunosuppressants Cyclosporin A (CsA) and FK506 (Schreiber and Crabtree, 1992). CsA and FK506 are not structurally similar and are both membrane permeant. In the cytosol, CsA and FK506 bind to intracellular receptors, the cyclophilins and immunophilins respectively. CsA/cyclophilin and FK506/immunophilin complexes bind to and inactivate CN. This is the basis of the immunosuppressive nature of these compounds; CN dependent transcription of multiple immune response genes is blocked in CsA/FK506 treated cells.

The many studies which have examined the regulation of NFAT sub cellular localisation reveal a number of required steps which are regulated in a receptor-driven fashion. However, the nature of these studies means that any consensus model for the process must contain elements drawn from a number of experimental systems and concerning multiple NFAT isoforms. It is by no means clear that the detailed aspects of NFAT regulation are conserved between cell systems or are similar for different NFAT family members. There are elements of the activation process which are common to all the systems examined to date. Numerous studies have contributed data allowing the model shown in Figure 7.1 to be drawn by Rao *et al* (1997), from whose review the figure is taken; (Loh *et al.*, 1996a; Loh *et al.*, 1996b; Park *et al.*, 1995; Ruff and Leach, 1995; Shaw *et al.*, 1995; Wesselborg *et al.*, 1996).

In resting cells NFAT proteins are phosphorylated and are exclusively resident in the cytosol. In T cells stimulated either through the T cell antigen receptor or the application of calcium ionophore NFAT is rapidly dephosphorylated and translocates to the nucleus. Dephosphorylated NFAT shows an increased affinity for DNA over that of phospho-NFAT isolated from resting cells (Jain *et al.*, 1995a). Clearly, the translocation of NFAT from cytosol to nucleus, where it can contact DNA, is a critical commitment step for the induction of NFAT/AP-1 transcriptional activity by immunoreceptors. The activation of NFAT is intimately connected to the status of the Calcineurin (CN) phosphatase, a major target for antigen receptor-derived calcium signals in immune cells.

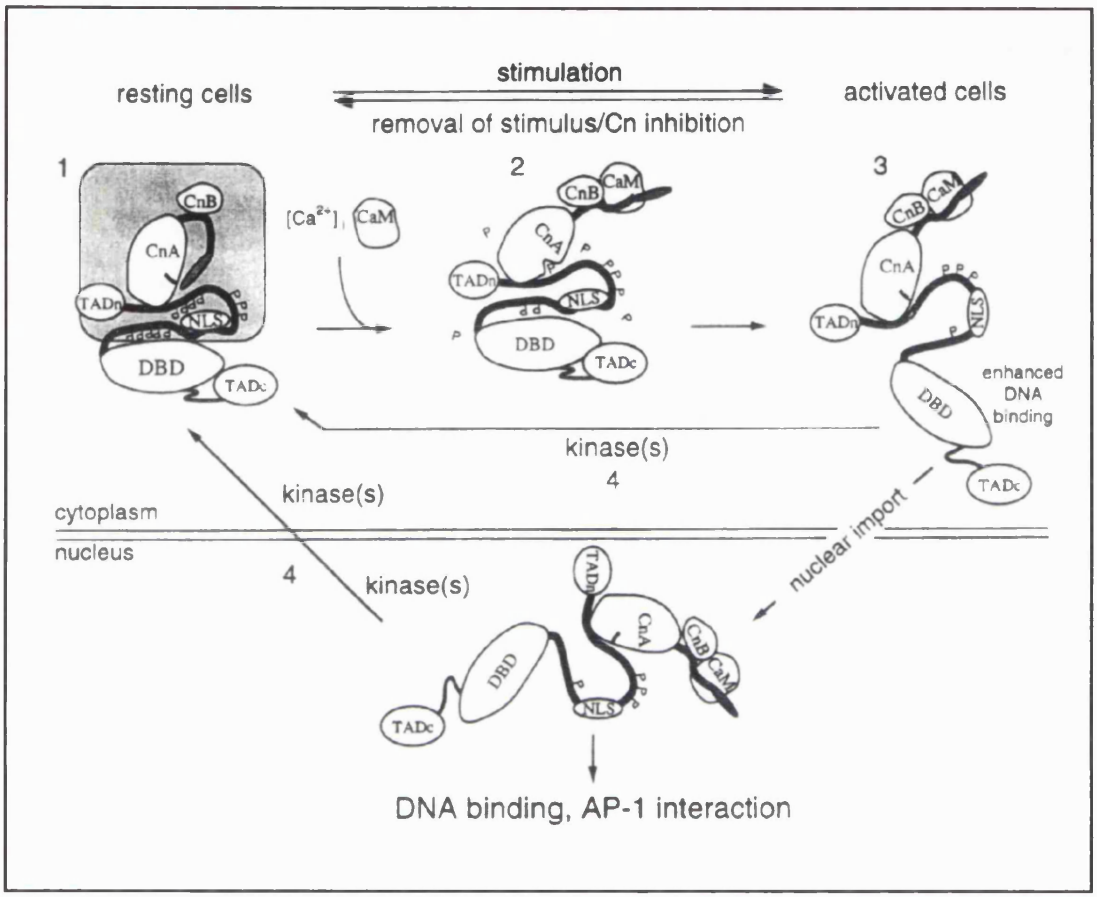


Figure 7.1 Stages in the NFAT activation process. Reproduced from Rao *et al* Annual Review of Immunology, 1997. (1) In resting cells cytosolic NFAT is complexed with Calcineurin (CN) A and B. The CNA autoinhibition is alleviated by the receipt of calcium/calmodulin signals (2) In the activated state CN is able to dephosphorylate NFAT at certain critical sites, causing a conformational change and exposing previously buried nuclear localisation signals (NLS) and DNA contact residues. Thus NFAT is able to interact with the nuclear import machinery and shows enhanced ability to bind DNA (3). Nuclear NFAT forms a complex with AP-1 and is transcriptionally active. Termination of calcium signaling or inhibition of CN leads to NFAT rephosphorylation by a nuclear NFAT kinase and nuclear export (4). TAD, transactivation domain; DBD, DNA binding domain; CaM, calmodulin;

In resting cells CN is inactive, with the autoinhibitory domain of CN blocking the enzyme's catalytic site. Elevation of intracellular free calcium ($[Ca^{2+}]_i$) triggers the binding of Calmodulin (CaM) to the autoinhibitory domain of CN, releasing autoinhibition by virtue of a conformational change. Active CN dephosphorylates NFAT, this step is permissive for nuclear import and will be discussed in more detail below. CN may accompany NFAT protein to the nucleus. Data supporting this concept comes from two sources:

First, Shibasaki *et al* examine the sub cellular localisation of NFAT4 and CN in the context of a hamster kidney fibroblast line (Shibasaki *et al.*, 1996). The data in this study show that elevated $[Ca^{2+}]i$ induces NFAT4 association with CN. This complex persists in the nucleus, where NFAT4 remains dephosphorylated. The authors propose that this continued dephosphorylation of NFAT4 is required for NFAT4 to remain transcriptionally active. An implication of this study is that CN is transported to the nucleus in order to counteract the activity of a putative nuclear NFAT kinase. Second there is the simple observation that each step of the activatory process can be blocked by CsA. These data suggest that the role of CN persists after the initial dephosphorylation of NFAT protein.

The activity of NFAT is intimately linked with the calcium signalling status of the cell. This has been exploited to illustrate the inherent reversibility of NFAT dephosphorylation and nuclear import. Sustained elevation of $[Ca^{2+}]i$ and hence activation of CN causes prolonged activation and nuclear localisation of NFATC1. If the calcium signal is removed, or CN inhibitory agents are introduced, then nuclear export of NFAT protein is rapid (Beals *et al.*, 1997a; Beals *et al.*, 1997b; Loh *et al.*, 1996b; Shaw *et al.*, 1995). Rephosphorylated NFAT protein is detectable in the cytosol within 5 min of addition of CsA to T lymphocytes previously treated with calcium ionophore. It is clear that the phosphorylation status of NFAT, which is regulated by the CN phosphatase and one or more opposing kinase activities, determines 1) the access of NFAT protein to the nucleus, 2) the duration of NFAT residency in the nucleus and therefore 3) commitment to transcriptional activation by nuclear NFAT protein.

7.1.3 NFAT dephosphorylation exposes buried Nuclear Localisation Sequences, permitting nuclear translocation.

NFAT proteins are composed of a number of discrete functional domains. The general domain structure of NFATs has been described in Chapter 6. An N-terminal regulatory domain contains several sequence motifs which are strongly conserved between NFAT family members. These include several potential phosphoacceptor sites for Proline directed kinases, organised into three serine/proline (S/P) box regions. The consensus motif found in these SP regions is SPxxSPxxSPxxxxx[D/E] (Beals *et al.*,

1997a; Ho *et al.*, 1995; Rao *et al.*, 1997). The regulatory region containing the SP boxes binds the CN phosphatase, is dephosphorylated in activated cells and deletion/truncation experiments reveal that it is absolutely required for NFAT translocation to the nucleus to be stimulated by CN (Luo *et al.*, 1996c).

The SP box region is extensive, containing multiple potential phosphoacceptor sites which are by definition possible CN target residues. The precise localisation of the residues dephosphorylated by CN has been studied in detail for NFATC1. Beales *et al* identified that Ala substitution of residues 172-176, 178-181 and 184-188 but not 191-194 of NFATC1 results in total nuclear accumulation of NFATC1 protein in the absence of CN activation (Beals *et al.*, 1997a). Moreover, these mutants were rendered insensitive to the action of FK506, indicating that rephosphorylation is required for exit from the nucleus.

Dephosphorylation of cytosolic NFAT protein is the major biochemical change measured for NFAT protein as a consequence of antigen receptor activation. CN acts directly upon phosho-NFAT, rather than via a CN-initiated protein phosphatase cascade. Such cascades, terminating in the activity of Protein Phosphatases 1 or 2A have been described for CN in cells of the central nervous system (Mulkey *et al.*, 1994). The effect of dephosphorylation upon NFAT protein seems to be twofold; 1) permitting nuclear import and 2) resulting in an increased affinity for DNA. Treatment of T cell extracts with CN or an alkaline phosphatase results in a marked increase in NFAT affinity for oligonucleotide DNA as measured in an EMSA system. Current models for the effect of dephosphorylation upon NFAT suggest that a marked conformational change in NFAT tertiary structure occurs. In the dephosphorylated state NFAT is postulated to have exposed Nuclear Localisation Sequences (NLS) which are accessible to the nuclear import machinery (Beals *et al.*, 1997a). In addition, a previously masked DNA recognition region in the Rel-homology DNA binding domain may be exposed.

The nuclear import machinery of eukaryotic cells is highly complex (reviewed in (Nigg, 1997). Nuclear Pore Complexes (NPCs) mediate the bi-directional transport of proteins, nucleic acids, ions and other species between cytosol and nucleus. The NPC is a multiprotein complex, composed of some 10^3 individual protein components (nucleoporins) many of which are present as multiple copies of the same molecule. The NPC diameter is approximately 120nm and its estimated Mr is 125kDa. Some regulatory aspects of the NPC will be discussed in more detail below, with particular reference to the potential roles of Ras family GTPases. However, in the context of NFAT phosphorylation status, it is clear that exposure of NLS is the initial step in the process of nuclear import. Exposed NLS allow a cargo protein to interact with the Importin α and β carrier molecules, which mediate translocation through the nuclear pore and are released and recycled once the cargo molecule has entered the nucleus.

NLS for proteins have been identified through a systematic approach of transferring amino acid clusters to cytosolic proteins and assaying whether nuclear import is induced. These NLS are mono- or bi-partite clusters of basic residues. The NFATC1 molecule found in T lymphocytes and mast cells contains four short regions of repeated basic amino acids which are putative monopartite NLS. Beals *et al.*, (1997a) *et al* tested these regions for activity as NLS by splicing them to the Sos molecule . Sos, as a GEF for the Ras GTPases, is targeted to the plasma membrane and is therefore normally exclusively cytosolic. Two of the putative NLS from NFATC1 were able to target Sos to the nucleus. The first of these incorporated residues 265-267, a region situated between the second and third SP boxes of the N-terminal regulatory region. The second NLS comprises residues 682-685, at the C-terminus of the Rel-homology DNA binding domain. The position of these is shown schematically in Figure 7.2. Both NLS have the core sequence KKRK, in common with numerous other NLS from proteins such as the SV40 T antigen.

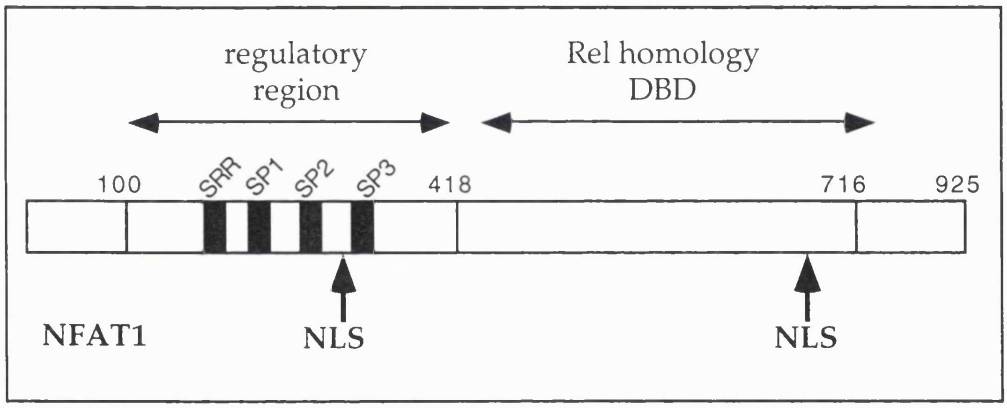


Figure 7.2. Domain structure of NFATC1 and position of NLS positively identified in Beals *et al.*, (1997a) . Short stretches of basic amino acids at the indicated positions can drive nuclear accumulation of a normally cytosolic molecule. NLS, Nuclear Localisation Signal; SRR, Serine Rich Region; SP, Serine/Proline; DBD, DNA binding domain.

Dephosphorylation is required for NFAT nuclear import and is the major biochemical difference observed in NFAT from stimulated and quiescent cells. Hence, it is likely that dephosphorylation is the modification required for exposure of these two NLS and commitment to the nuclear import machinery. A model for this exposure is shown in Figure 7.3. On the basis of primary sequence, it is unlikely that phosphate residues are adjacent to NLS and directly mask them. It is more likely that the intrinsic conformational change caused by dephosphorylation moves the KKRK sequences away from the environment of the SP boxes. It is apparently necessary for both NLS to be exposed and both must be disrupted by mutation in order to render NFAT constitutively cytosolic.

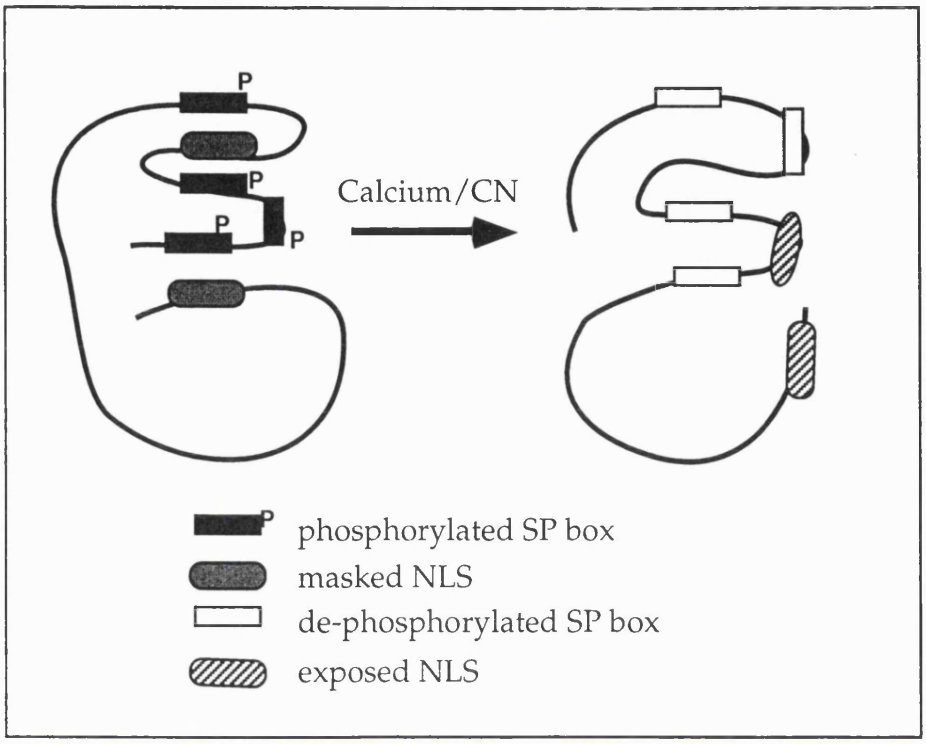


Figure 7.3. NFAT dephosphorylation causes a conformational change which exposes previously buried NLS. This model is taken from (Beals *et al.*, 1997a). Cytosolic NFAT is phosphorylated at the SP boxes. The action of calcineurin (CN) dephosphorylates these sites, the resultant conformational change alleviates the masking of NLS which are then accessible to the nuclear import machinery.

7.1.4 The activity of CN is opposed by one or more NFAT kinases.

The phosphorylation status of NFAT proteins determines their subcellular localisation and hence availability to mediate transcriptional activation. The enzyme responsible for the dephosphorylation of NFAT, CN, is well studied. The availability of CsA and FK506, which inhibit the activity of CN when bound to their immunophilin ligands, has enabled detailed examination of the role of NFAT dephosphorylation in its activation pathway. However, it is clear that NFAT proteins must be substrates for one or more NFAT kinases, which are responsible for 1) the constitutive phosphorylation of NFAT in the cytosol of resting cells and 2) the re-phosphorylation of nuclear NFAT in response to termination of calcium signalling or the addition of CsA/FK506, leading to nuclear export.

NFAT kinases have not been exhaustively identified in any cell system. NFAT is a substrate for kinase activity in two distinct subcellular compartments, the cytosol and the nucleus. Hence two models can be envisaged for NFAT phosphorylation; 1) NFAT is a substrate for a priming kinase activity in the cytosol and for a distinct nuclear resident kinase. 2) a single NFAT kinase is partitioned across the nuclear

membrane. A recent report has identified one kinase activity purified from brain extracts which phosphorylates NFATC1. Beals *et al* (1997b) show that two species from brain extract can be purified on the basis of their ability to phosphorylate NFATC1 derived fusion proteins. The data show that Protein kinase A (PKA) and the Glycogen synthase kinase-3β (GSK-3β) could be responsible for NFATC1 phosphorylation. Specific inhibition of PKA later showed that PKA is unlikely to be the NFAT kinase in either brain or lymphocyte extracts. In contrast, GSK-3β was found to be of direct relevance to NFATC1 phosphorylation status, although these experiments were performed in a heterologous Cos cell system. Functionally, over-expression of GSK-3β caused increased nuclear export of NFATC1 in a manner dependent on GSK-3β kinase activity.

It is clear that GSK-3β and an as yet uncharacterised priming kinase activity synergise to phosphorylate NFATC1 upon the sites involved in calcium dependent nuclear import. These experiments have not been performed in antigen receptor stimulated immune cells and hence it is not clear if GSK-3β is controlling NFAT localisation in lymphoid cells. It would seem necessary to perform immunodepletion or dominant inhibition studies of GSK-3β to ask if its function is required for NFAT nuclear import in an immune cell system. Stimulation of peripheral T cells via PdBu/Ionomycin does lead to rapid inactivation of GSK-3 β (Welsh *et al.*, 1996). If this is reflected in pathways regulated by the T cell antigen receptor, then these data would lead to an attractive model where elevated $[Ca^{2+}]_i$ causes NFAT nuclear localisation by 1) activating the NFAT phosphatase, CN, and 2) down-regulating the opposing NFAT kinase GSK-3β and hence suppressing re-phosphorylation and nuclear export. GSK-3β is partitioned between the cytosol and nucleus and could therefore be correctly compartmentalised to be solely responsible for NFAT phosphorylation. However, immunodepeletion of GSK-3β from brain extracts does not fully prevent NFATC1 phosphorylation although GSK-3β protein was undetectable by Western blot in depleted extracts. This suggests that there is an important priming kinase activity in the cytosol which is distinct from GSK-3β. Hence it is possible that cytosolic and nuclear NFAT are targets for different kinases.

7.1.5 Signalling pathways required for nuclear import of NFAT protein.

The regulation of NFAT/AP-1 complex transcriptional activity has been studied in some detail. Typically the contribution of a signalling pathway to NFAT/AP-1 activity can be assessed using dominant active or inhibitory mutants of candidate signalling proteins in the context of an antigen receptor stimulated immune cell. However, from these studies it is not easy to identify whether the target of the signal is the CN/NFAT interaction or the AP-1 dimer. Knowledge concerning regulation of the

CN/NFAT interaction is currently focused on the generation of elevated $[Ca^{2+}]_i$ in response to antigen receptor ligation.

Ligation of antigen receptors typically results in a biphasic modulation of $[Ca^{2+}]i$ levels, comprising a rapid transient influx of calcium from the environment, followed by a sustained elevation in $[Ca^{2+}]i$ due to the release of calcium from intracellular stores. There is considerable evidence that these different types of calcium flux can give rise to distinct biological outcomes. Timmerman *et al* differentiate between induction of events such as muscle contraction, secretion, adhesion and synaptic transmission by transient calcium flux and the sustained calcium signals which are necessary for a cell to commit to proliferation or differentiative programmes (Timmerman *et al.*, 1996). Sustained high concentrations of $[Ca^{2+}]i$ are apparently required to maintain NFAT localisation to the nucleus and hence transcriptional activity. This reflects the need for continued CN activity to oppose a nuclear NFAT kinase. In a B lymphocyte system, Dolmetsch *et al* demonstrate that as described above, NFAT is selectively activated by sustained elevation of $[Ca^{2+}]i$ (Dolmetsch *et al.*, 1997). In contrast, the transcriptional regulators NFκB and Jun kinase are activated by a large transient rise in $[Ca^{2+}]i$. Hence, nuclear factors differentiate between types and durations of calcium signal generated by antigen receptors.

It is not clear whether receptor derived signals other than elevation of $[Ca^{2+}]i$ regulate the CN/NFAT interaction and hence nuclear import. At the level of nuclear import itself there are demonstrated requirements for the activity of the GTPase Ran (Nigg, 1997). The GTP- and GDP-loaded forms of Ran bind selectively to components of the transport machinery and control protein-protein interactions which are important for cargo recognition and trafficking. The Ran GAP and GEF are localised to the cytosol and nucleus respectively. Ran in the cytosol is primarily GDP-loaded whilst that in the nucleus is GTP-loaded and active. Signals which regulate Ran are poorly characterised. Ran is an integral part of the nuclear import machinery which functions constitutively to transport hundreds of proteins and ribonucleoprotein particles every minute. It seems unlikely that this process is a specific target for signals generated in response to ligation of antigen receptors. However, there is published biochemical evidence that a GTPase other than Ran is important for nuclear import (Sweet and Gerace, 1996). It remains to be seen whether this represents 1) another GTPase activity which is part of the basic transport machinery or 2) an indicator of a GTPase signalling requirement, which is potentially regulatable by transmembrane receptors.

There is another potential level of regulation for the nuclear import of NFAT proteins which has not been fully investigated and may encompass a role for GTPases of the Ras family. The role of the actin cytoskeleton in NFAT translocation has not been examined, although induction of certain NFAT regulated cytokine genes is

sensitive to Cytochalasin D, which inhibits actin polymerisation. Both direct and indirect roles for components of the actin cytoskeleton in the control of NFAT subcellular localisation can be envisaged: 1) Directly, the relocation of a large pool of protein across the nuclear membrane may require morphological changes dependent on actin and 2) Indirectly, structures such as focal adhesions represent foci of signalling proteins interacting with adhesion molecules (eg. integrins) which may be involved in basal calcium signalling. Levels of [Ca2+]i set by these non-antigen receptors may be important in establishing the subsequent antigen sensitivity of N FAT nuclear import.

7.1.6 Aims.

Nuclear import of NFAT is a critical commitment step for the subsequent activity of NFAT/AP-1 transcription factor complexes. The activity of the calcium regulated phosphatase Calcineurin (CN) is required for NFAT dephosphorylation and hence for nuclear import. The identity of the NFAT kinase which is responsible for the constitutive phosphorylation and cytosolic retention of NFAT is less well understood. It is clear that the regulation of NFAT phosphorylation and sub cellular localisation is an important facet of the manner in which antigen receptors regulate cytokine gene induction. In the mast cell system the regulation of NFAT sub cellular localisation has not been examined. In Chapter 6 data was presented showing the requirement for Ras and Rac-1 in the regulation of NFAT/AP-1 transcriptional activity. Therefore the aims of the work presented in this chapter were as follows:

1) To establish an assay system for the sub cellular localisation of NFATC1 protein in RBL2H3 mast cells.

2) To examine FcεR1 regulation of NFATC1 localisation in RBL2H3.

3) To ask if FcεR1 regulation of Ras family GTPases is important in the control of NFATC1 sub cellular localisation in this system.

7.2 RESULTS.

7.2.1 A Green Fluorescent Protein marker for NFATC1 localisation in RBL2H3 mast cells.

An assay was required to follow the sub cellular localisation of NFATC1 in mast cells. Limited amounts of a poor antiserum for NFATC1 were available, precluding a conventional immunofluorescence approach. Since one of the eventual aims of this work was to assay the effects of GTPase mutants upon NFAT sub cellular localisation, a transient transfection/GFP marker approach was attempted. In such an assay system the use of an excess of regulator over reporter plasmid removes the necessity for sorting of transfected cells. The marker used was the Green Fluorescent Protein (GFP) molecule. Construction of the reporter plasmid peGFP-NFATC1 and its derivative peGFP-NFATC1ΔRel was carried out by Dr Edward McKenzie of the Yamanouchi Research Institute, Oxford. These constructs were generously made available for the NFATC1 translocation assay to be established as described below.

The GFP of *Aequorea victoria* is a 25kDa protein with two absorbance peaks at 395 and 475nm. Excitation at 395nm leads to emission at 508nm. The GFP chromophore is formed by the cyclisation of 3 residues; Ser65, Tyr66 and Gly67. Cyclisation is completed by dehydrogenation of the Tyr residue. GFP technology has found a wide number of applications, including the tagging of proteins to assay their sub cellular localisation. Several reviews summarise the published applications of GFP (Cubitt *et al.*, 1995; Heim and Tsien, 1996). In the work presented below GFP was fused to the full-length NFATC1 molecule. This NFATC1 marker construct (NFATC1-GFP) was used in transient transfection of RBL2H3 and is shown schematically in Figure 7.4.

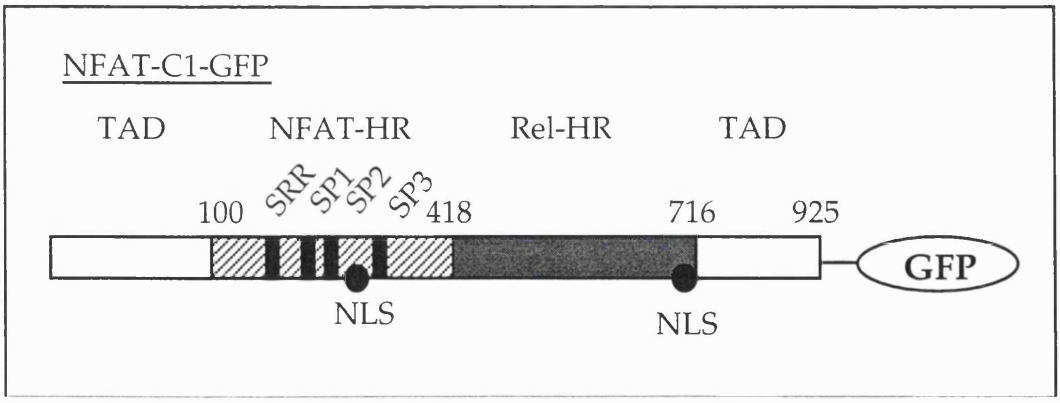


Figure 7.4. NFATC1-GFP marker construct. Schematically shown here, the full-length NFATC1 molecule was fused to the GFP reporter by cloning into the Clontech peGFP-1 vector. TAD, transactivation domain; HR, homology region; NLS, nuclear localisation sequence; GFP, green fluorescent protein; SRR, serine rich region;, SP, Ser/Pro box.

Initial experiments established the transfection conditions for use of the NFATC1-GFP reporter. RBL2H3 were transfected with varying amounts of plasmid DNA and allowed to express the fusion protein at 37°C for the indicated times. The data in Figure 7.5 show that the optimum expression levels were reached at 6h recovery after transfection of 8μg NFATC1-GFP reporter per 10^7 RBL2H3. At higher DNA levels or longer recovery times no increase in transfection efficiency was obtained. However, over-expression of high levels of the GFP tagged protein was found to result in visible nuclear loading with GFP. Optimal expression levels gave less bright but exclusively cytoplasmic expression of the GFP fusion proteins.

time (h) \ μg DNA	4	8	12	16	20
2	0/0	**0/0**	3/0	4/0	8/1
4	0/0	**18/2**	25/4	20/6	19/6
6	0/0	**36/2**	30/11	27/8	29/14
8	5/0	**40/7**	36/18	25/14	36/16
12	4/0	**29/11**	39/15	29/11	41/19

Figure 7.5. Transfection conditions for use of NFATC1-GFP reporter in RBL2H3. 10^7 RBL2H3 per point were transfected with the indicated amounts of reporter DNA. Cells were plated onto glass coverslips and recovered at 37°C for the time indicated in DMEM/10% FCS. After fixation and mounting, 50 cells per coverslip were scored for GFP expression. The percentage of cells expressing GFP is indicated (left figure). Of these the percentage with significant nuclear localisation of the NFATC1-GFP protein were assessed (right figure).

The following assay strategy was developed. RBL2H3 were transiently transfected with NFATC1-GFP reporter, alone or in combination with an expresssion vector for a regulatory signalling protein. Cells were recovered for 5h, and primed with IgE anti-DNP for a further hour. Non-FcεR1 stimulated cells were recovered for 6h in total. After recovery cells were stimulated. At the end of the stimulation medium was replaced sequentially with warm (37°C) PBS followed by 4% paraformaldehyde fixative. Fixed cells were either mounted directly or stained with Propidium iodide (PI) to visualise nuclei and then mounted. The slides bearing the mounted coverslips were

randomised and then examined by fluorescence microscope. Cells were scored for GFP localisation as predominantly cytosolic, nuclear or both. Replicate coverslips were produced per condition and 100 cells per coverslip were scored.

7.2.2 FcεR1 and calcium signals regulate NFATC1 sub cellular localisation in RBL2H3.

Signals generated by the T cell antigen receptor have been shown to cause NFAT protein translocation from cytosol to nucleus. FcεR1 ligation results in the expression of multiple genes which are under the control of NFAT/AP-1 transcription factor complexes. Therefore the location of NFATC1-GFP in resting and FcεR1 stimulated RBL2H3 was examined. The data in Figure 7.6a show that in resting RBL2H3 (control), NFATC1-GFP is predominantly cytosolic and does not co-localise with the nuclear stain Propidium Iodide (red, right panels). After 30 min exposure to crosslinking antigen (KLH-DNP), NFATC1-GFP is exclusively nuclear. The kinetics of this response are shown in Figure 7.6b, NFATC1-GFP is exported from the cytosol, with a concomitant increase in nuclear GFP levels. At 30 min stimulation, typically 80-90% cells exhibit exclusively nuclear NFATC1-GFP. Over longer timecourses NFATC1-GFP was slowly exported from the nucleus, with approximately 50% cells showing predominantly nuclear localisation of NFATC1-GFP at 60 min stimulation (data not shown). These data show that FcεR1 stimulation of RBL2H3 mast cells results in the rapid nuclear import of NFATC1-GFP protein.

As described in the Introduction, the calcium regulation of the Calcineurin phosphatase (CN) is a major intracellular signalling pathway affecting NFAT phosphorylation and hence sub cellular localisation. Data from the B and T cell systems suggest that a sustained elevation in intracellular free calcium levels ($[Ca^{2+}]i$) is required for NFAT relocalisation to the nucleus and to maintain its presence in this compartment. Two pharmacological agents were used to elevate $[Ca^{2+}]i$ and their effects on the sub cellular localisation of NFATC1 were examined.

Capacitative calcium entry, influx occurring in response to depletion of intracellular stores, has been demonstrated in various cell types including the RBL2H3 (Lee and Oliver, 1995). The sarcoplasmic or endoplasmic reticulum calcium-ATPase (SERCA) is normally responsible for pumping calcium into intracellular stores. Thapsigargin is an inhibitor of the SERCA pump. Thapsigargin (TG) therefore causes sustained increases in $[Ca^{2+}]i$ by activating the capacitative calcium entry pathway. The calcium ionophore Ionomycin causes a rapid transient rise in $[Ca^{2+}]i$ followed by a sustained plateau of elevated $[Ca^{2+}]i$. This response is attributable to both release of calcium from internal stores and influx from the environment. There is evidence that FcεR1 stimulation of RBL2H3 causes an increase in $[Ca^{2+}]i$ which is composed of

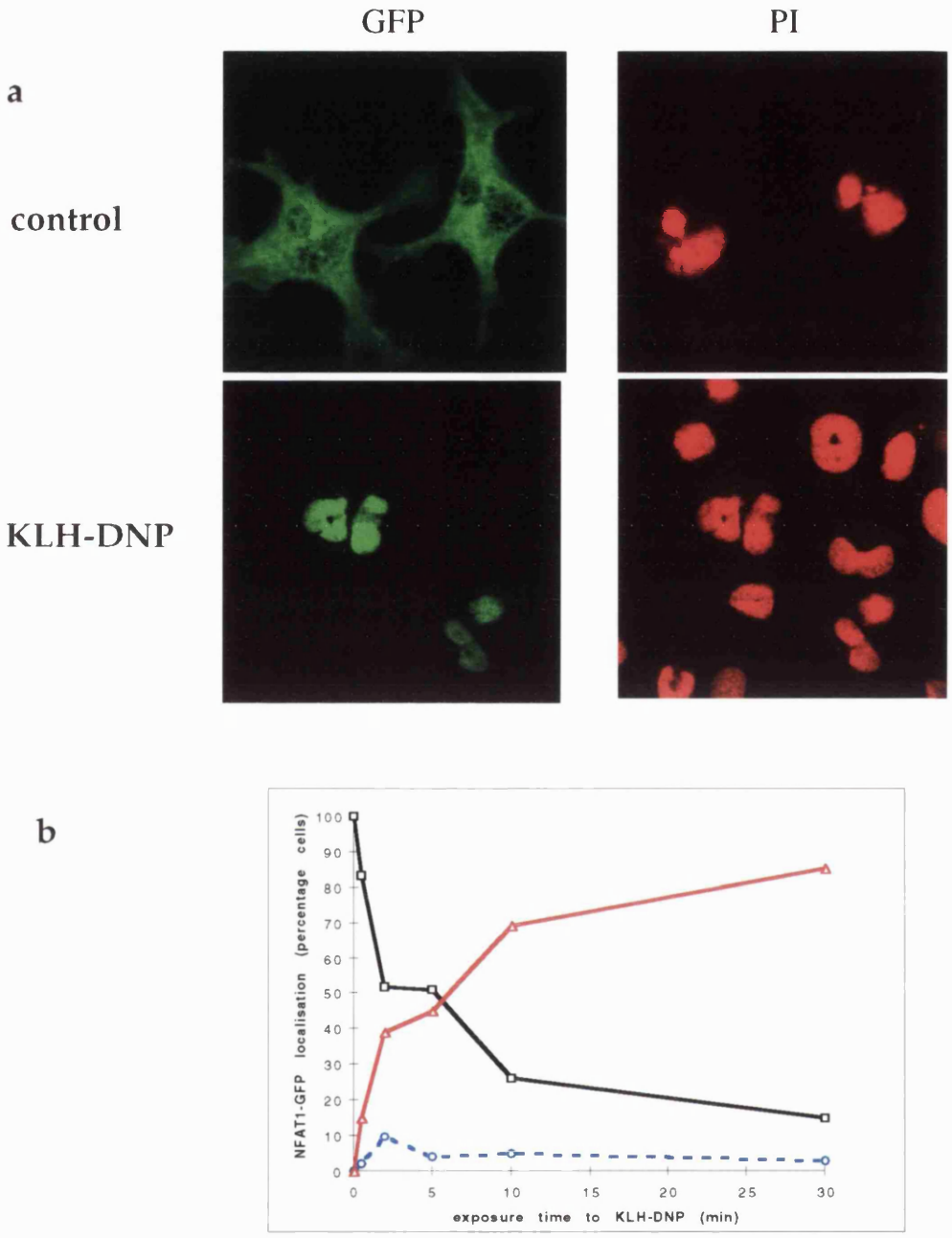


Figure 7.6. FcεR1 antigenic crosslinking causes NFATC1-GFP nuclear import in RBL2H3. RBL2H3 were transfected with 8μg NFATC1-GFP reporter and recovered for 6h on glass coverslips in complete medium at 37oC. Cells were primed and stimulated with 500ng/ml KLH-DNP for 30 min (a) or for the indicated times (b). Cells were fixed and mounted as described in Materials and Methods. a) Cells were co-stained with propidium iodide to delineate the nucleus (red, right panels). b) Cells were scored for the predominant localisation of GFP protein using a fluorescence microscope. Replicate fields of 100 cells/coverslip were counted per timepoint. Samples were scored in a blind-coded fashion.

both receptor mediated calcium influx and capacitative calcium influx pathways (Lee and Oliver, 1995). The effects of both Ionomycin and Thapsigargin on NFATC1-GFP localisation in RBL2H3 were examined.

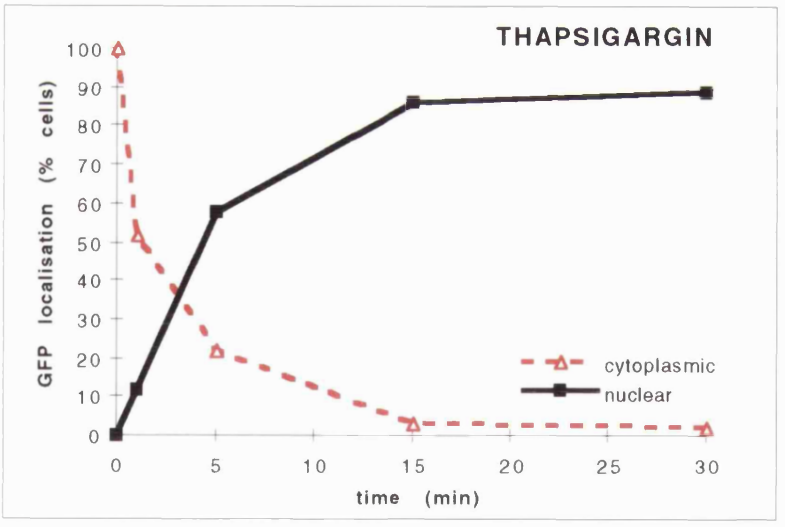
The data in Figure 7.7a show that the application of 0.1μM Thapsigargin (TG) causes NFATC1-GFP relocalisation from the cytosol to the nucleus over a 30 minute timecourse. NFATC1-GFP nuclear import is essentially complete within 15 minutes of addition of TG. The calcium ionophore Ionomycin also causes NFATC1-GFP nuclear import in a manner indistinguishable from that seen with TG (Figure 7.7b). Phorbol esters do not cause any mobilisation of $[Ca^{2+}]i$, or by themselves cause any relocalisation of NFATC1-GFP (data not shown). However, it is clear that phorbol ester does enhance ionophore induced transcriptional activity of NFAT/AP-1 CAT reporter genes. The effect of combining phorbol ester (PdBu) and calcium ionophore upon NFATC-1GFP nuclear import was examined. The data in Figure 7.7c show that PdBu does not significantly enhance NFATC1-GFP nuclear import in response to calcium ionophore. The kinetics of nuclear import were slightly accelerated over that observed with ionophore alone, but this effect was slight and its significance is not clear.

Figure 7.7. Capacitative calcium influx and calcium ionophore cause NFATC1-GFP nuclear import in RBL2H3. RBL2H3 were transfected with 8μg NFATC1-GFP reporter and recovered for 6h on glass coverslips in complete medium at 37°C. Cells were stimulated with either 0.1μM Thapsigargin (a) 0.5μM Ionomycin alone (b) or in combination with 50ng/ml PdBu (c) for the indicated times. Cells were fixed and mounted as described in Materials and Methods. Cells were scored for the predominant localisation of GFP protein using a fluorescence microscope. Replicate fields of 100 cells/coverslip were counted per timepoint. Samples were scored in a blind-coded fashion.

7.2.3 FcεR1 stimulated NFATC1-GFP nuclear import is a calcineurin activated, Cyclosporin A sensitive process.

Fusion to the 25kDa GFP molecule is a major modification of the NFATC1 protein. An important consideration with this type of experiment is that one may be modifying the behaviour of the protein of interest as well as rendering it more amenable to study. Control experiments were performed to assess whether the behaviour of the NFATC1-GFP protein is comparable to that expected for the endogenous NFATC1 molecule. As described in the introduction to this chapter, dephosphorylation of NFAT by the Calcineurin (CN) phosphatase is permissive for nuclear import and hence for assembly of productive NFAT/AP-1 complexes. CN activity is the target of the immunosuppressive drugs Cyclosporin A (CsA) and FK506. Hence the application of CsA blocks NFAT nuclear import and the induction of NFAT/AP-1 dependent

a


THAPSIGARGIN
GFP localisation (% cells)
100
90
80
70
60
50
40
30
20
10
0
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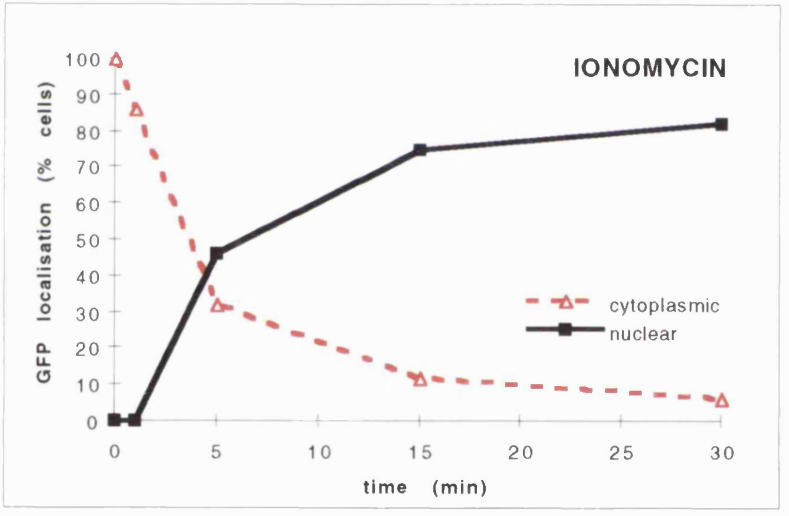
5
10
15
20
25
30
time (min)
cytoplasmic
nuclear

b


IONOMYCIN
GFP localisation (% cells)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
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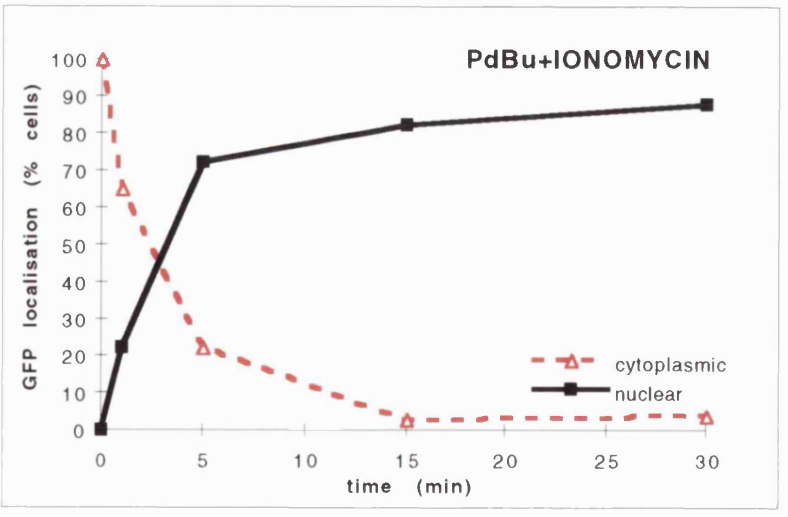
25
30
time (min)
cytoplasmic
nuclear

c


PdBu+IONOMYCIN
GFP localisation (% cells)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
time (min)
cytoplasmic
nuclear

transcriptional events. Moreover, in cells where nuclear accumulation of NFAT has been induced, application of CsA causes the rapid export of NFAT from the nucleus to the cytosol.

In order to be considered as a good model for endogenous NFAT regulation, NFATC1-GFP nuclear import should therefore be positively regulated by CN and sensitive to the action of CsA. Figure 7.8 shows the results of experiments testing these criteria. In control cells, singly transfected with reporter, NFATC1-GFP is predominantly nuclear after 30 min FcεR1 stimulation. In cells co-transfected with an activated mutant of CN (CNM), NFATC1-GFP is localised to the nucleus in the absence of FcεR1 stimulation. Conversely, pre-treatment of RBL2H3 with CsA ablates FcεR1 induced NFATC1-GFP nuclear import. These data suggest that NFATC1-GFP is subject to the same characteristic regulation as endogenous NFAT.

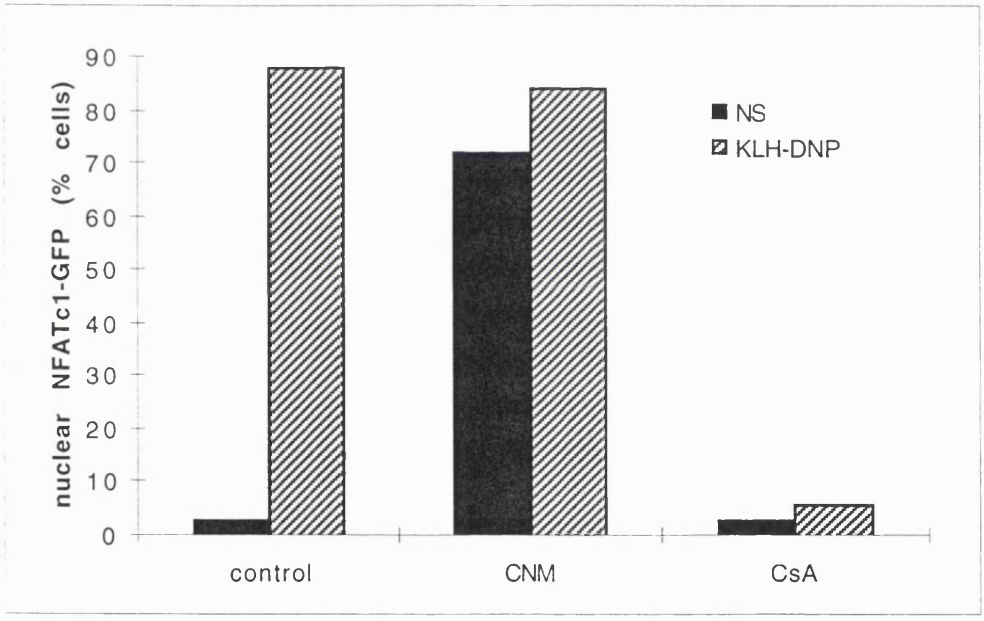


Figure 7.8. FcεR1 induction of NFATC1-GFP nuclear import is CN regulated and CsA sensitive. RBL2H3 were transfected with 8μg NFATC1-GFP reporter alone or in combination with 20μg activated CN plasmid (CNM) and recovered for 6h on glass coverslips in complete medium at 37°C. Cells were primed and stimulated with 500ng/ml KLH-DNP for 30 min in the absence or presence of 50nM Cyclosporin A (CsA). Cells were fixed, mounted and scored for predominant localisation of GFP protein as described in Materials and Methods.

The data in Figure 7.8 establish two important points; 1) that the NFATC1-GFP reporter is likely to closely model the behaviour of endogenous NFAT and 2) that the approach of co-transfecting a plasmid bearing a regulatory molecule is feasible in this assay system.

7.2.4 Dominant inhibition of the Ras GTPase does not affect NFATC1-GFP nuclear import.

Previous experiments have shown that Rac-1 and Ras function are required for FcεR1 induction of NFAT/AP-1 transcriptional activity. Accordingly, possible roles for Ras and Rac-1 in the regulation of NFATC1 nuclear import in RBL2H3 were examined. The N17 mutants of these two GTPases act to sequester guanine nucleotide exchange factor (GEF) from endogenous pools of the GTPase, maintaining the GTPase in its GDP-bound, inactive, state. Co-transfection of these mutants removes the ability of the FcεR1 to signal via the relevant GTPase.

The data in Figure 7.9 show the kinetics of NFATC1-GFP nuclear import in response to antigenic crosslinking of the FcεR1 and Ionomycin. In these experiments cells were either singly transfected with NFATC1-GFP reporter or co-transfected with the RSV N17 Ras plasmid. The data show that the presence of N17 Ras does not affect the kinetics or magnitude of the NFATC1-GFP response to FcεR1 stimulation (Figure 7.9a). Moreover, dominant inhibition of Ras activity does not inhibit NFATC1-GFP nuclear import in response to treatment with the calcium ionophore, Ionomycin (Figure 7.9b). Parallel control experiments were performed to check that the RSV N17 Ras plasmid was functional and that N17 Ras protein was being expressed. The Elk-1 transcription factor is a target for Ras signals in RBL2H3 (see Chapter 4). The data in Figure 7.9c show that equivalent levels of the RSV N17 Ras plasmid to those used in the NFATC1-GFP reporter transfections were able to severely compromise FcεR1 induction of Elk-1 transcriptional activity. These data suggest that there is no problem with expression of the N17 Ras protein in RBL2H3 to levels where Ras signalling pathways are effectively blocked. The lack of effect of the N17 Ras on NFATC1-GFP nuclear import suggests that this is not a Ras dependent process.

7.2.5 Dominant inhibition of the Rac GTPase severely compromises NFATC1-GFP nuclear import.

The FcεR1 cannot drive NFAT/AP-1CAT reporter gene activity in cells bearing the N17 Rac-1 mutant. These data suggest that Rac-1 is involved in a critical signalling pathway regulating the activity of this transcriptional complex. The experiments presented here asked whether Rac-1 signals regulate the sub cellular localisation of NFATC1 protein in RBL2H3. Cells were transfected with NFATC1-GFP reporter either alone or in combination with pEF N17 Rac-1, the dominant inhibitory mutant of the Rac-1 GTPase.

Figure 7.10a shows laser scanning confocal images of RBL2H3 stimulated by antigenic crosslinking of the FcεR1. The sub cellular localisation of NFATC1-GFP reporter (green, left panels) was compared to the nuclear staining of Propidium iodide (red, right panels). The data show that in FcεR1 stimulated cells transfected with

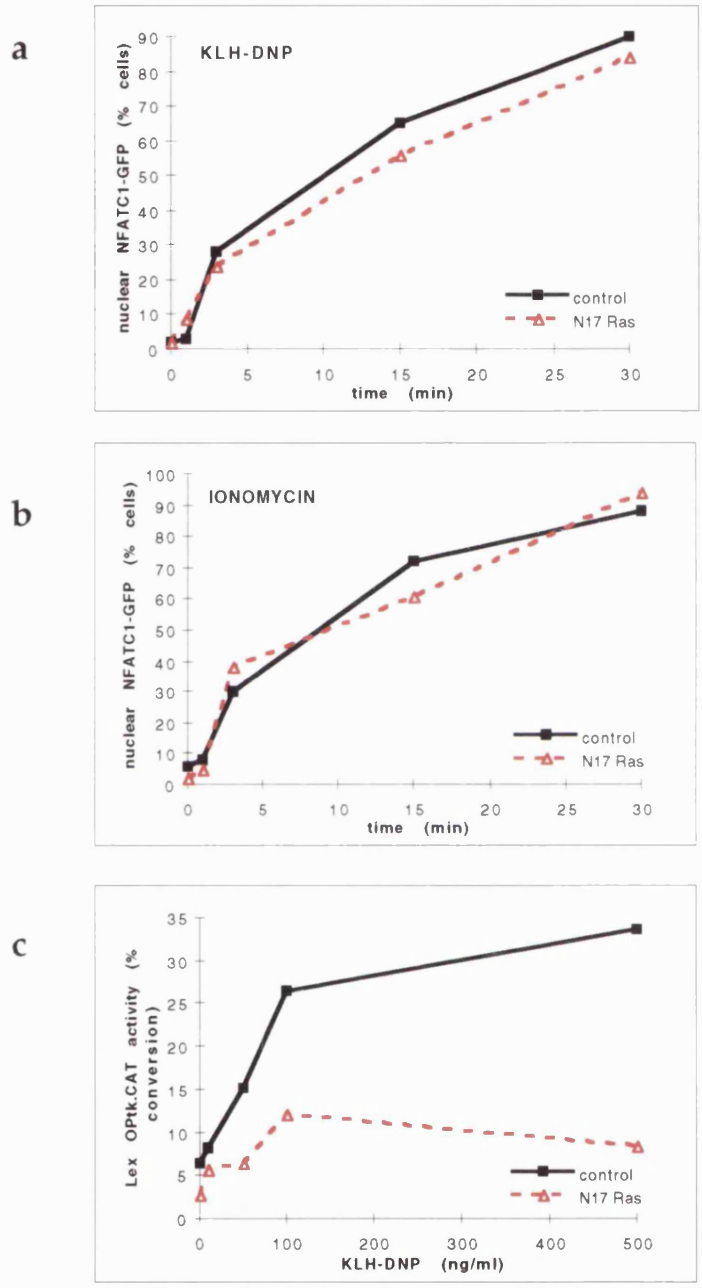


Figure 7.9. Dominant inhibition of Ras does not affect NFATC1-GFP nuclear import. (a,b) 10^7 RBL2H3 were transfected with 18µg NFATC1-GFP alone or in combination with 20µg RSV N17 Ras. Cells were seeded onto glass coverslips, recovered for 6h and stimulated as indicated using 500ng/ml KLH-DNP or 500ng/ml ionomycin. Stimulations were stopped using 4% paraformaldehyde at the indicated timepoints. NFATC1-GFP nuclear localisation was assayed as described. (c) 10^7 RBL2H3 were transfected with 12µg pEF LexAOP tk.CAT/6µg pEF LexA Elk-1 alone or in combination with 20µg RSV N17 Ras. Cells were recovered for 6h before priming, stimulation and CAT assay as described.

NFATC1-GFP alone, GFP and PI co-localise. This indicates that FcεR1 stimulation has caused NFATC1-GFP nuclear import as expected. In cells co-transfected with N17 Rac-1 (Figure 7.10b), FcεR1 stimulation does not result in nuclear accumulation of NFATC1-GFP. Rather, in N17 Rac-1 expressing cells NFATC1-GFP is effectively retained in the cytosol. The data in Figure 7.10c show a kinetic experiment examining NFATC1-GFP nuclear accumulation in the absence and presence of co-transfected N17 Rac-1. Across a population of cells, NFATC1-GFP nuclear import is severely abrogated in N17 Rac-1 expressing cells. After 30 min stimulation via the FcεR1, only 15% of cells bearing N17 Rac-1 have nuclear NFATC1-GFP, compared with 85% control cells. These data suggest that Rac-1 signals are required for FcεR1 control of NFATC1-GFP nuclear import.

The data in Figure 7.7b showed that elevation in $[Ca^{2+}]i$ caused by the application of the calcium ionophore Ionomycin is sufficient to induce NFATC1-GFP nuclear import. The effect of N17 Rac-1 upon Ionomycin induced NFATC1-GFP nuclear import was examined. The data in Figure 7.10d show that N17 Rac-1 does not markedly inhibit the nuclear accumulation of NFATC1-GFP in response to Ionomycin. These data are representative of four experiments. The maximal inhibition of Ionomycin induced NFATC1-GFP nuclear import observed on any occasion with N17 Rac-1 was approximately 25%. In contrast, N17 Rac-1 consistently inhibited FcεR1 induction of this response by 75-80%. Thus the calcium flux induced by Ionomycin is apparently able to overcome the requirement for Rac-1 in the control of NFATC1-GFP localisation. As will be discussed below, it is not clear whether these data reflect quantitative and/or qualitative differences in the calcium fluxes elicited by ionomycin and the FcεR1.

7.2.6 Dominant active Rac-1 potentiates the FcεR1 induction of NFATC1-GFP nuclear import.

The data in Figure 7.10 have shown that Rac-1 signals are required for FcεR1 induction of NFATC1-GFP nuclear import in response to antigen. These data were obtained using a dominant inhibitory form of the Rac-1 GTPase to block signalling through that pathway. Since Rac-1 is neccessary for FcεR1 induction of NFATC1-GFP nuclear import, the question arises as to whether Rac-1 signals are sufficient to induce this response. The V12 mutant of Rac-1 is resistant to the activity of GTPase Activating Proteins (GAPs) and is therefore retained in the GTP-bound, active state. Accordingly, the effect of V12 Rac-1 co-transfection on NFATC1-GFP sub cellular localisation was examined.

The data in Figure 7.11a show that FcεR1 stimulation of RBL2H3 causes NFATC1-GFP export from the cytosol in a manner dose-dependent upon the amount of crosslinking antigen applied. In control cells, singly transfected with NFATC1-

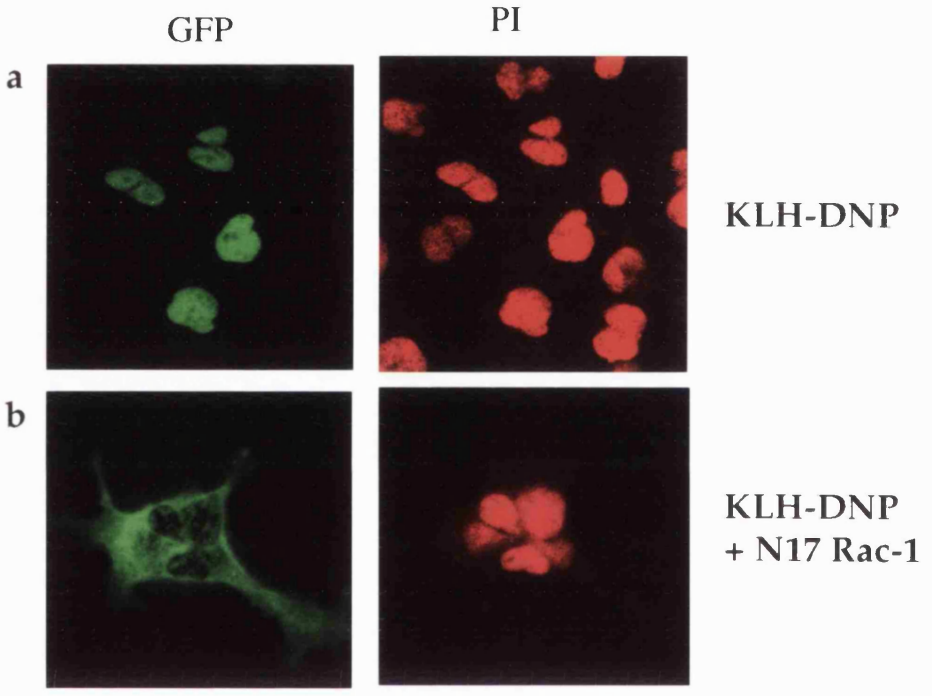
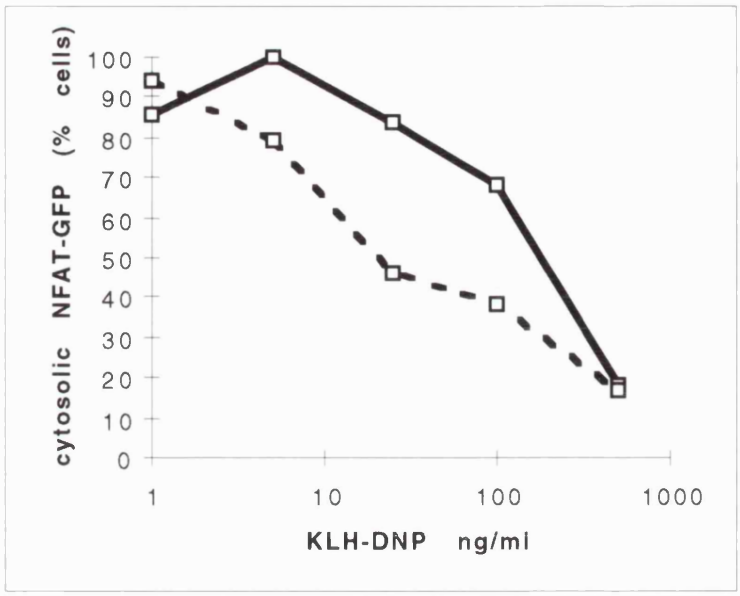


Figure 7.10. (a,b) N17 Rac-1 prevents FcεR1 induced NFATC1-GFP nuclear import. RBL2H3 were transfected with 8μg NFATC1-GFP reporter alone (a) or in combination with 20μg pEF N17 Rac-1 (b) . Cells were recovered for 6h on glass coverslips before IgE priming and stimulation with 500ng/ml KLH-DNP. Cells were fixed, mounted and localisation of GFP protein was visualised as described in Materials and Methods.

a



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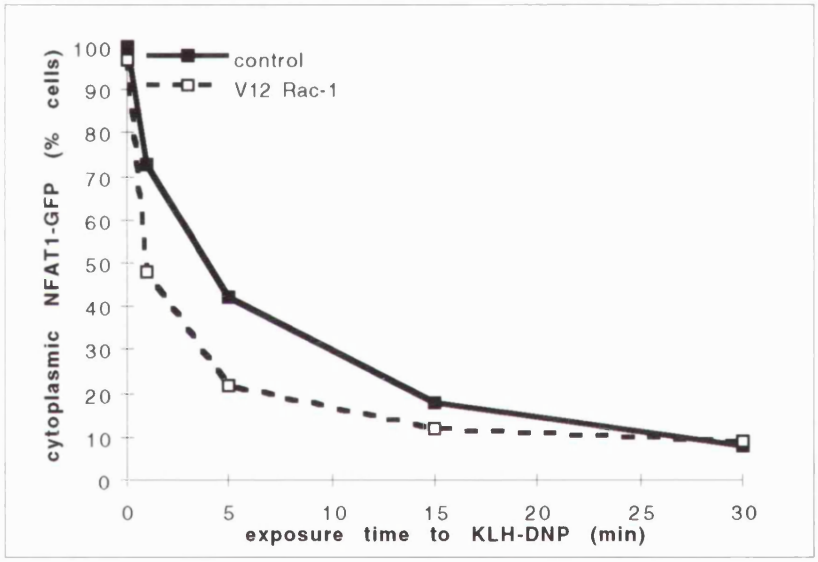


Figure 7.11. (a,b) Effect of V12 Rac-1 upon NFATC1-GFP nuclear import in RBL2H3. RBL2H3 were transfected with 8µg NFATC1-GFP reporter alone or in combination with 20µg pEF V12 Rac-1 . Cells were recovered for 6h on glass coverslips before IgE priming and stimulation for 30 min with the indicated concentrations of KLH-DNP (a) or stimulation with 500ng/ml KLH-DNP for the indicated times (b). Cells were fixed, mounted and scored for predominant localisation of GFP protein as described in Materials and Methods.

GFP alone, a dose of 20ng/ml KLH-DNP causes 50% maximal export of NFATC1-GFP from the cytosol. In cells bearing V12 Rac-1, there is a marked shift in the dose response for FcεR1 stimulation of NFATC1-GFP translocation to the nucleus. In this case, 50% maximal import is achieved at approximately 200ng/ml KLH-DNP. V12 Rac-1 does not induce NFATC1-GFP nuclear accumulation in the absence of other stimuli, in contrast with the effect of this mutant on the transcriptional activity of NFAT/AP-1.

Constitutive activation of Rac-1 signalling using V12 Rac-1 also causes an acceleration in the kinetics of FcεR1 induced NFATC1-GFP nuclear import. The data in Figure 7.11b show that in the presence of V12 Rac-1 the early phase of FcεR1 mediated NFATC1-GFP nuclear import is accelerated. Although it is slight, this acceleration was reproducible between experiments. The data in Figure 7.12 show that across four experiments the effect of V12 Rac-1 was to enhance FcεR1 mediated NFATC1-GFP nuclear import.

Experiment	Nuclear NFATC1-GFP after 5 min KLH-DNP (% cells)		% increase with V12 Rac-1
	control	+V12 Rac-1	
1	41	59	18
2	36	64	28
3	38	62	24
4	43	57	14

Figure 7.12. V12 Rac-1 reproducibly accelerates NFATC1-GFP nuclear import in response to FcεR1 stimulation of RBL2H3. These data summarise the results of four separate experiments. The percentage of cells with predominantly nuclear NFATC1-GFP after 5 min exposure to KLH-DNP is shown, from cells either singly transfected with reporter alone (control) or co-transfected with pEF V12 Rac-1. In cells bearing V12 Rac-1 there was a 14-28% increase in the number of cells with nuclear NFATC1-GFP localisation at 5 min stimulation.

The effect of V12 Rac-1 upon the FcεR1 dose response for NFATC1-GFP nuclear import is clear. V12 Rac-1 also slightly accelerates the kinetics of FcεR1 mediated NFATC1-GFP nuclear import, although this is unlikely to be a highly significant effect in terms of induction of NFAT/AP-1 transcriptional activity. However, the inhibition of NFATC1-GFP nuclear import by N17 Rac-1 and conversely the enhancement of this response by V12 Rac-1 suggest that Rac-1 is involved in the FcεR1 regulation of NFATC1 sub cellular localisation. This is apparently a role specific to Rac-1. Dominant inhibition of Ras, the only other Ras family GTPase to be required for NFAT/AP-1 transcriptional activation by the FcεR1, does not affect NFATC1-GFP nuclear import. There has previously been no description in any system of a role for non-calcium/CN signals in the regulation of NFAT sub cellular localisation. Therefore the data presented here potentially represent a novel and interesting role for a Ras family GTPase.

7.2.7 Rac-1 control of NFATC1-GFP sub cellular localisation is not connected to Rac-1 regulation of the actin cytoskeleton.

There is an established body of work upon the role of Rac/Rho family GTPases in orchestration of actin cytoskeleton rearrangements. Stimulation of growth factor receptors in fibroblasts leads to the formation of membrane ruffles and stress fibres. There is extensive data detailing the critical role of Rac-1, Rho and CDC42Hs in control of these events (Nobes and Hall, 1995; Tapon and Hall, 1997). Rac-1 is required for the formation of membrane ruffles in fibroblasts. Rac-1 activation results in Rho GTP loading and activation. Rho.GTP regulates the formation of focal adhesions and the generation of bundled actin stress fibres. FcεR1 stimulation of the RBL2H3 mast cell line is known to result in marked rearrangements of the actin cytoskeleton. Stimulated RBL2H3 form actin plaques/focal adhesions at their adherent surface and membrane ruffles at the top of the cell (Norman *et al.*, 1994; Pfeiffer and Oliver, 1994). Hence potentially Rac-1 driven cytoskeletal rearrangements are a consequence of FcεR1 antigenic crosslinking.

FcεR1 mediated NFATC1-GFP nuclear import involves the translocation of a considerable pool of protein from the cytosol to the nucleus. It is possible that this type of event requires a contribution from the actin cytoskeleton in the form of morphological changes in the RBL2H3. Since there is evidence that these changes can be under the control of Rac-1 they are candidates for the basis of the role for Rac-1 in regulation of NFATC1 sub cellular localisation. Accordingly, the role of actin cytoskeleton rearrangements in NFATC1-GFP nuclear import was examined.

FcεR1 stimulation of RBL2H3 causes the formation of actin structures such as stress fibres, focal complexes and membrane ruffles (Figure 7.13a, left panel), which are visualised using Rhodamine-phalloidin staining for polymerised actin.

Cytochalasin D is an inhibitor of actin polymerisation. This compound prevents FcεR1 induced cytoskeletal changes such as the induction of focal complex formation and stress fibres. (Figure 7.13a right panel). RBL2H3 were transfected with NFATC1-GFP reporter and incubated either with vehicle or Cytochalasin D for 30 min prior to stimulation via IgE/FcεR1. The data in Figure 7.13b show that application of Cytochalasin D does not affect FcεR1 induced NFATC1-GFP nuclear import. In both control and Cytochalasin D treated cells FcεR1 stimulation leads to the rapid export of NFATC1-GFP from the cytosol and its concomitant accumulation in the nucleus. These data suggest that rearrangements of the actin cytoskeleton are in fact not required for FcεR1 regulation of NFAT nuclear import. Therefore the basis of Rac-1 involvement in the regulation of NFATC1 sub cellular localisation is distinct from the published roles for Rac-1 in the regulation of the actin cytoskeleton. In summary of the data presented so far, it is clear that a novel role for Rac-1 has been described. Rac-1 activity is a previously unrecognised player in the regulation of NFAT sub cellular localisation, either at the level of general nuclear transport or as a selective regulator of NFAT. Moreover, the mechanism by which Rac-1 accomplishes this function is separate from the established Rac-1 regulation of the actin cytoskeleton.

7.2.8 Dominant inhibition of Rac-1 does not cause a general blockade in nuclear import.

In the presence of N17 Rac-1, the FcεR1 is unable to drive the nuclear import of NFATC1-GFP. It is possible that this is either 1) an effect of Rac-1 upon the general nuclear import machinery or 2) a role for Rac-1 in the specific regulation of NFAT sub cellular localisation. As described in the Introduction, dephosphorylation of NFAT proteins is thought to permit nuclear import due to a conformational change which exposes previously buried Nuclear Localisation Sequences (NLS) (Beals *et al.*, 1997a). At the level of the nuclear pore complex (NPC) there is a requirement for the GTPase activity of Ran and another, as yet uncharacterised GTP binding protein (Sweet and Gerace, 1996). Accordingly, an assay was established to test if inhibition of Rac-1 signalling affected NFAT translocation at the level of the nuclear import machinery.

A truncated version of the NFATC1-GFP marker was generated by Dr Edward McKenzie, Yamanouchi Research Institute, Oxford. This construct, NFATC1ΔRel-GFP, was generously made available for the following experiments and is shown schematically in Figure 7.14 in comparison with the full-length reporter used above. NFATC1ΔRel-GFP lacks the N-terminal regulatory domain and the two transactivation

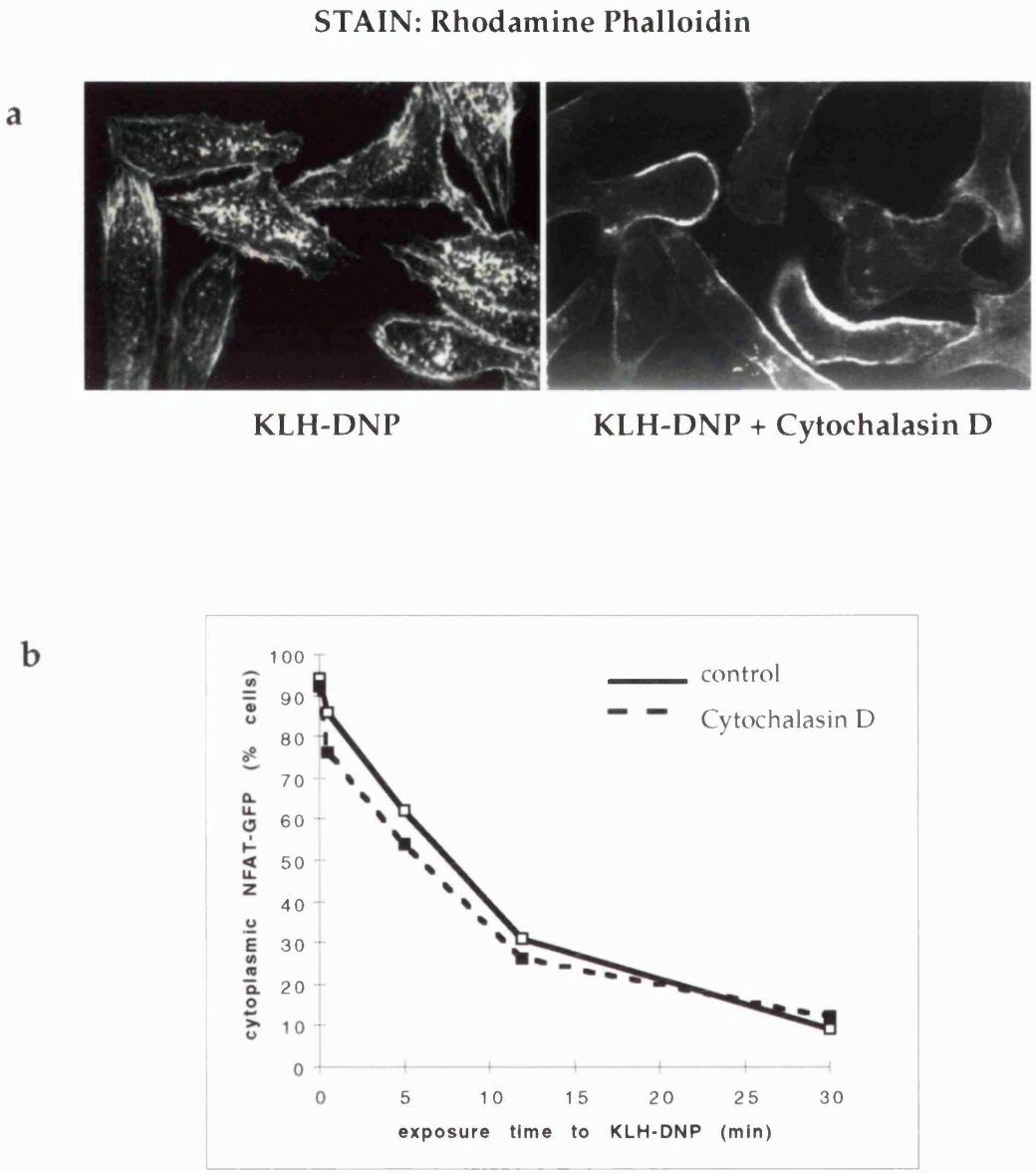


Figure 7.13. Cytochalasin D does not affect NFATC1-GFP nuclear import. (a) RBL2H3 were seeded onto glass coverslips. Cells were IgE primed and stimulated with 500ng/ml KLH-DNP for 30min in the absence (left) or presence (right) of 500nM Cytochalasin D. Cells were fixed, permeabilised with Tritonx100 and polymerised actin was visualised using 0.25µg/ml Rhodamine-Phalloidin. (b) RBL2H3 were transfected with 8µg NFATC1-GFP reporter. Cells were recovered for 6h on glass coverslips before IgE priming and stimulation for the indicated times with 500ng/ml KLH-DNP in the absence or presence of 500nM Cytochalasin D.

domains at the N- and C-termini of the full-length protein. The phosphorylated SP motifs within the N-terminal regulatory region are thought to mask NLS. Therefore it was expected that the NFATC1ΔRel-GFP would have constitutively exposed NLS. This truncated protein would therefore be independent of any regulation of NFAT tertiary structure leading to exposure of buried NLS in the full-length protein, but would still be reliant on the NPC for nuclear import.

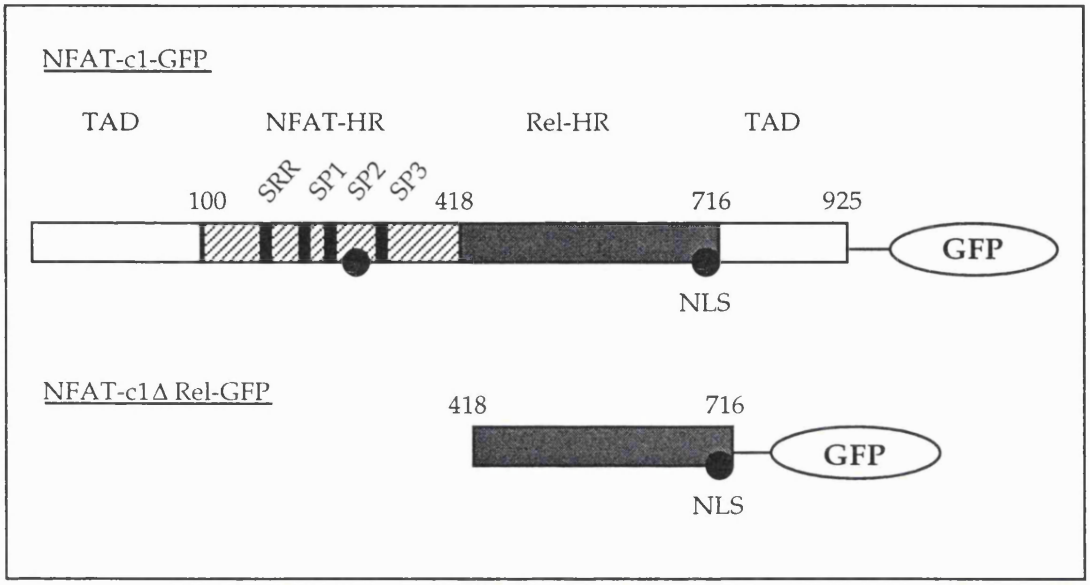


Figure 7.14. NFATC1-GFP and NFATC1ΔRel-GFP reporter gene constructs. Full length and truncated versions of NFATC1 were fused to the GFP molecule. NFATC1ΔRel-GFP lacks the N-terminal regulatory domain containing the SP box phosphoacceptor sites and is therefore thought to have a constitutively exposed NLS.

The NFATC1ΔRel-GFP was transfected into RBL2H3 and its sub cellular localisation was assayed under a number of conditions. Firstly, it was shown that in resting RBL2H3 NFATC1ΔRel-GFP is constitutively localised to the nucleus (Figure 7.15a). The data in Figure 7.15b show that N17 Rac-1 expression does not affect the constitutive nuclear localisation of the truncated NFATC1ΔRel-GFP protein. Figure 7.15c and d compare the subcellular localisation of NFATC1-GFP and NFATC1ΔRel-GFP in control and FcεR1 stimulated RBL2H3 in the absence and presence of co-transfected N17 Rac-1.

The effect of the NFATC1ΔRel truncation has been to effectively divorce the localisation of the GFP-tagged molecule from NFAT regulatory signalling pathways. This is shown in Figure 7.16, since application of Cyclosporin A does not prevent nuclear import or promote nuclear export of NFATC1ΔRel-GFP. This is in marked contrast to the effect of CsA on nuclear translocation of the full-length NFATC1-GFP.

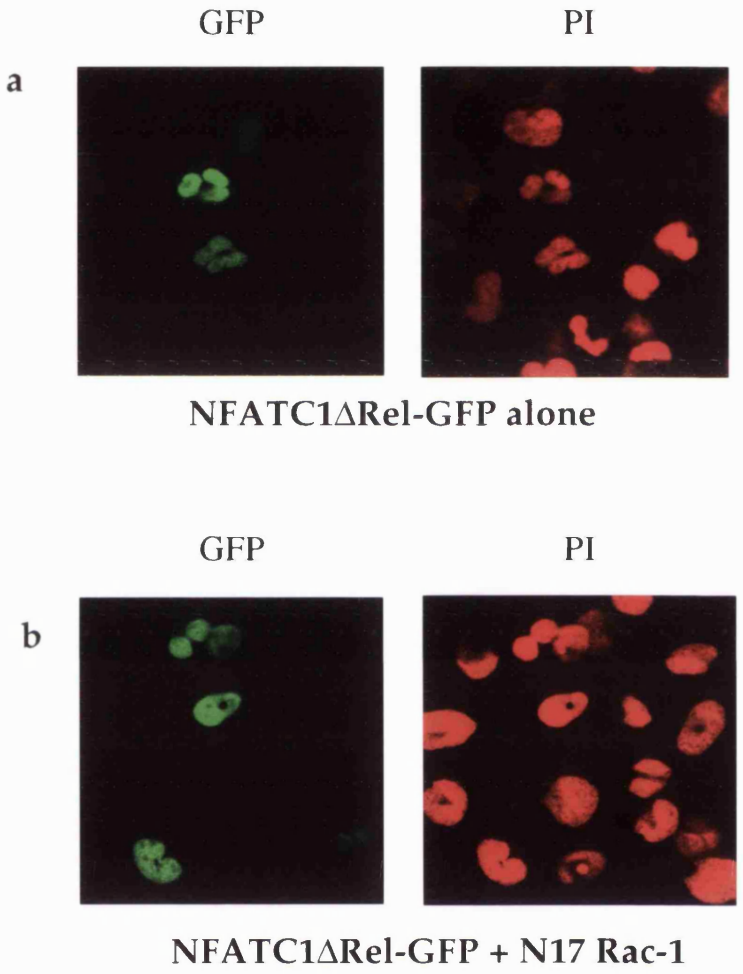


Figure 7.15. (a,b) N17 Rac-1 does not affect the subcellular localisation of NFATC1ΔRel-GFP. RBL2H3 were transfected with 10μg NFATC1ΔRel-GFP reporter alone (a) or in combination with 20μg pEF N17 Rac-1 (b) Cells were recovered for 6h on glass coverslips before IgE priming and stimulation for 30 min with 500ng/ml KLH-DNP. Cells were fixed and nuclear material was stained using propidium iodide (red, right panels). GFP localisation (green, left panels) and PI staining were imaged as described in Materials and Methods.

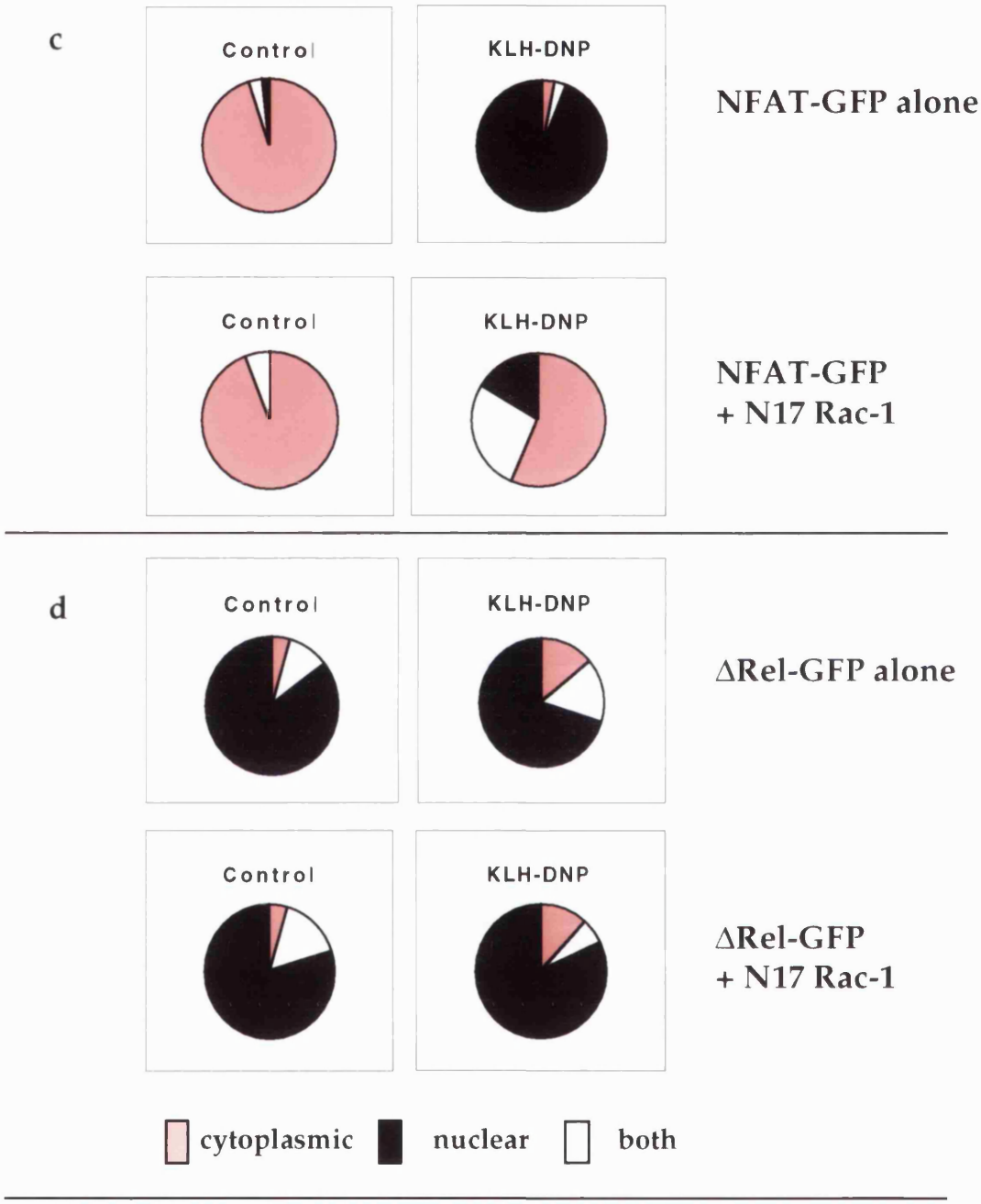


Figure 7.15. (c,d) N17 Rac-1 does not affect the subcellular localisation of NFATC1ΔRel-GFP. RBL2H3 were transfected with 8μg NFATC1-GFP (c) reporter or 10μg NFATC1ΔRel-GFP reporter (d) alone or in combination with 20μg pEF N17 Rac-1. Cells were recovered for 6h on glass coverslips before IgE priming and stimulation for 30 min with 500ng/ml KLH-DNP. Cells were fixed, mounted and scored for predominant localisation of GFP protein as described in Materials and Methods.

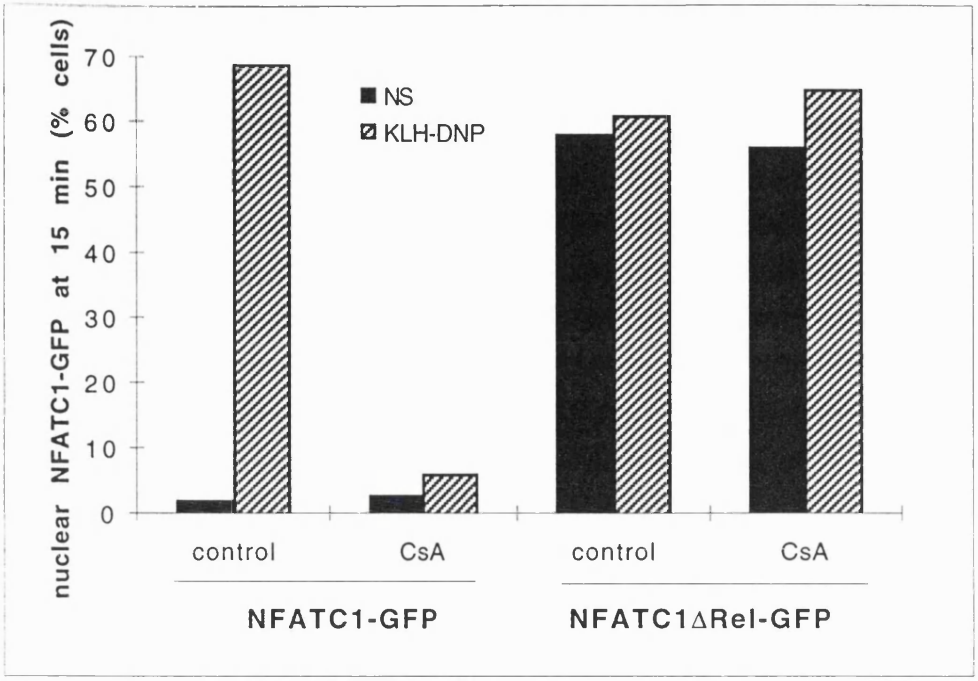


Figure 7.16. Constitutive nuclear localisation of NFATC1ΔRel-GFP is insensitive to Cyclosporin A. RBL2H3 were transfected with 8μg NFATC1-GFP reporter or 10μg NFATC1ΔRel-GFP and recovered for 6h on glass coverslips in complete medium at 37°C. Cells were primed and stimulated with 500ng/ml KLH-DNP for 30 min in the absence or presence of 50nM Cyclosporin A (CsA). Cells were fixed, mounted and scored for predominant localisation of GFP protein as described in Materials and Methods.

Rac-1 regulation of NFATC1 sub cellular localisation is unlikely to be a reflection of a role for this GTPase in some facet of the general nuclear import machinery. This is because 1) N17 Rac-1 does not affect the constitutive (but still NPC mediated) nuclear localisation of NFATC1ΔRel-GFP and 2) the delivery of a strong, sustained calcium signal by Ionomycin can overcome the N17 Rac-1 block of NFATC1-GFP nuclear import. The data obtained using NFATC1ΔRel-GFP also imply that the signal blocked by expression of N17 Rac-1 is affecting some part of the NFATC1 molecule other than the Rel-homology DNA binding domain. The NFATC1ΔRel-GFP protein lacks the two terminal putative transactivation domains and the N-terminal regulatory domain known to contain the Ser/Pro (SP) box motifs. There is considerable evidence that these motifs contain the phosphoacceptor sites which are targets for the NFAT kinase and CN phosphatase. Therefore it is reasonable to suggest at this point that the target for the Rac-1 signalling pathway which regulates NFAT sub cellular localisation could be within this known regulatory domain.

7.2.9 A gel-shift assay for NFATC1-GFP dephosphorylation shows that N17 Rac-1 targets NFAT phosphorylation status.

NFAT proteins are constitutively serine phosphorylated at multiple phosphoacceptor sites in resting cells. Upon cell stimulation, calcium dependent activation of the Calcineurin (CN) phosphatase results in the dephosphorylation of a number of these sites and permits nuclear import as described above. The dephosphorylation event can be visualised for endogenous NFAT as a shift to a higher mobility form of the protein in SDS-PAGE (Beals *et al.*, 1997a; Shaw *et al.*, 1995). An assay was established for NFATC1-GFP phosphorylation status in RBL2H3. The aim of this type of experiment was to examine the effect of co-transfection of N17 Rac-1 upon the mobility of the NFATC1-GFP protein in SDS-PAGE. Since this is a co-transfecion experiment it was necessary to assay the phosphorylation state of the reporter protein rather than endogenous NFATC1.

NFATC1-GFP is phosphorylated when in the cytosol and when dephosphorylated is in the nucleus. Therefore, it was necessary to establish extraction conditions which would enable the production of total cell lysate for Western analysis, i.e. containing both nuclear and cytosolic proteins. Extraction of proteins from the nucleus is not always straightforward. For the experiment in Figure 7.17, RBL2H3 were transfected with the NFATC1ΔRel-GFP construct, in this case used as a marker of nuclear localised, GFP-tagged, protein. Replicate aliquots of cells were used to prepare nuclear and cytosolic fractions. The nuclear fraction was prepared using various concentrations of NaCl in the lysis buffer as indicated. The data show that high (0.42M) concentrations of NaCl are required to effectively purify NFATC1ΔRel-GFP from the cell nucleus. Therefore in subsequent assays, total cell lysate was produced using a buffer containing 0.42M NaCl to ensure effective nuclear extraction.

The data in Figure 7.18 show anti-GFP Western analysis of Ionomycin-induced mobility shift of NFATC1-GFP in SDS-PAGE. In resting cells the NFATC1-GFP migrates as a number of species (90-130kDa), reflecting differently phosphorylated forms of the protein. In Ionomycin treated cells, NFATC1-GFP is markedly shifted to a higher mobility form, reflecting CN dependent dephosphorylation. Treatment with CsA effectively blocks Ionomycin induced mobility shift of the NFATC1-GFP protein. These data suggest that 1) it is feasible to assay the phosphorylation status of NFATC1-GFP in this fashion and 2) that the GFP-tagged version of NFATC1 behaves in a CN regulated, CsA sensitive manner in this biochemical assay, similar to that expected for the endogenous molecule.

The gel-shift assay described above was used to examine the effect of N17 Rac-1 upon FcεR1 regulation of NFATC1-GFP phosphorylation status. RBL2H3 were transfected with NFATC1-GFP reporter alone or in combination with pEF N17 Rac-1. The data in Figure 7.19 show that antigenic crosslinking of the FcεR1 results

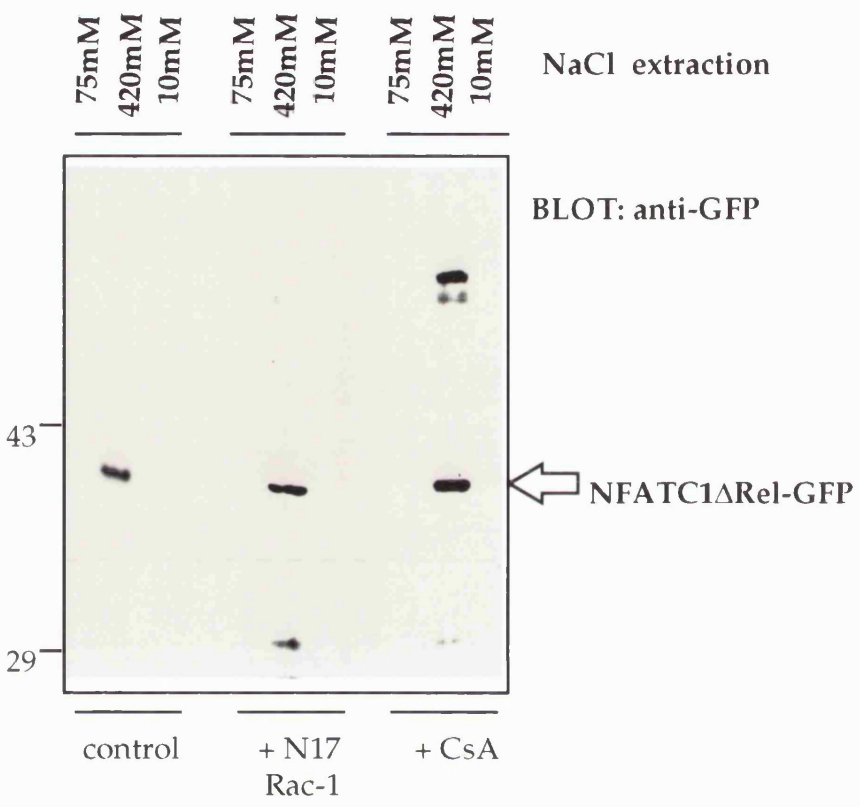


Figure 7.17. NaCl extraction of nuclear localised NFATC1ΔRel-GFP from RBL2H3. 10^7 RBL2H3 per point were transfected with 10µg NFATC1ΔRel-GFP alone or in combination with 20µg pEF N17 Rac-1 and recovered for 6h in the absence or presence of 50nM Cyclosporin A (CsA). Cells were harvested and lysed for Western analysis in a buffer containing 1% NP-40, 50mM HEPES pH7.4, 10mM iodoacetamide, 1mM PMSF and the indicated concentration of NaCl. Proteins were acetone precipitated and resolved by 10% SDS-PAGE before anti-GFP Western analysis using 1µg/ml anti-GFP.

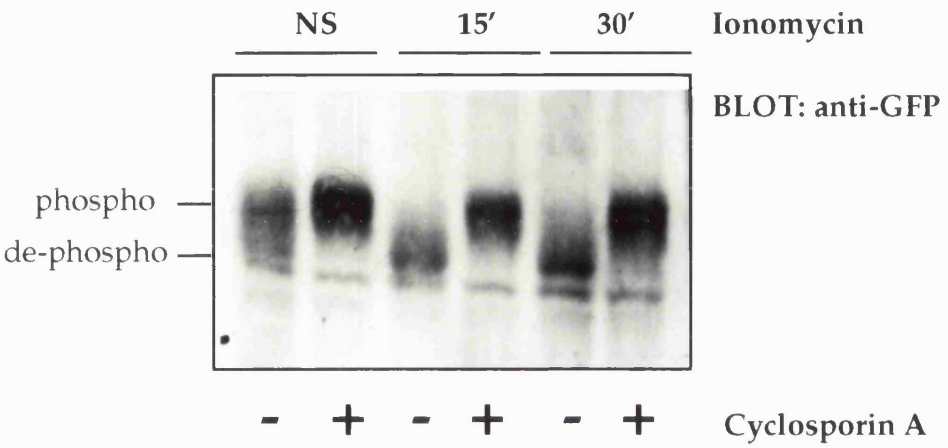


Figure 7.18. Ionomycin causes a mobility-shift in NFATC1-GFP, indicating dephosphorylation. 10^7 RBL2H3 per point were transfected with 10µg NFATC1-GFP and recovered for 6h in the absence or presence of 50nM Cyclosporin A (CsA), before stimulation for the indicated times with 500ng/ml Ionomycin. Cells were harvested and lysed for Western analysis in a buffer containing 1% NP-40, 50mM HEPES pH7.4, 10mM iodoacetamide, 1mM PMSF and 0.42M NaCl. Proteins were acetone precipitated and resolved by 6% SDS-PAGE before anti-GFP Western analysis using 1µg/ml anti-GFP.

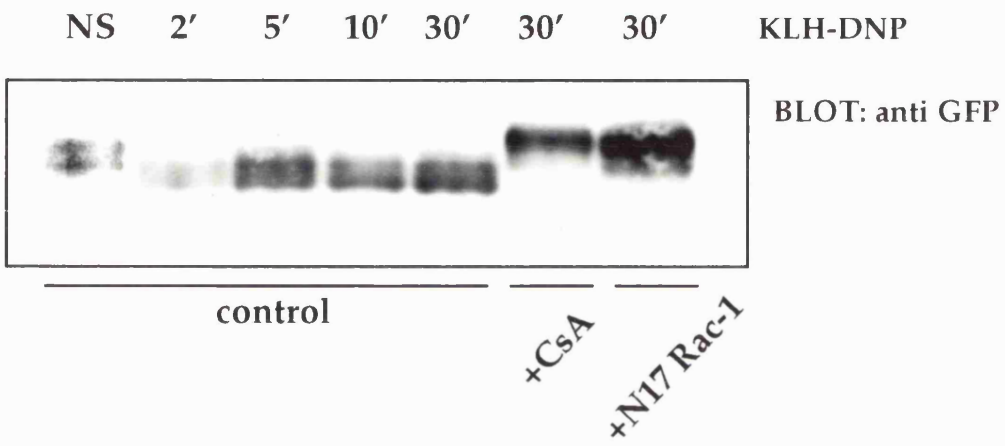


Figure 7.19. FcεR1 mediated NFATC1-GFP dephosphorylation is inhibited by N17 Rac-1 or CsA. 10^7 RBL2H3 per point were transfected with 10μg NFATC1-GFP alone or in combination with 20μg pEF N17 Rac-1 and recovered for 6h in the absence or presence of 50nM Cyclosporin A (CsA), before IgE priming and stimulation for the indicated times with 500ng/ml KLH-DNP. Cells were harvested and lysed for Western analysis in a buffer containing 1% NP-40, 50mM HEPES pH7.4, 10mM iodoacetamide, 1mM PMSF and 0.42M NaCl. Proteins were acetone precipitated and resolved by 6% SDS-PAGE before anti-GFP Western analysis using 1μg/ml anti-GFP.

in increased mobility of NFATC1-GFP, reflecting dephosphorylation by CN. Dephosphorylation is evident within 2 min exposure to crosslinking antigen and is sustained for 30 min. Pretreatment of RBL2H3 with CsA prevents FcεR1 mediated dephosphorylation of NFATC1-GFP, in this case NFATC1-GFP is retained in the low mobility, phosphorylated form. Figure 7.19 also shows the effect of N17Rac-1 expression upon FcεR1 stimulated NFATC1-GFP mobility shift. In cells bearing N17 Rac-1, NFATC1-GFP is effectively retained in the lower mobility form. Hence N17 Rac-1 expression mimics the effect of CsA, in that it blocks NFATC1-GFP dephosphorylation.

The data in Figure 7.20 show that whilst N17 Rac-1 effectively blocks FcεR1 mediated NFATC1-GFP dephosphorylation, it does not affect the mobility shift induced by Ionomycin. In contrast, CsA is able to overcome both antigen and ionophore stimulation to retain NFATC1-GFP in the phosphorylated, low mobility form. These data are consistent with the observations from the NFATC1-GFP sub cellular localisation assay, where N17 Rac-1 blocks FcεR1 but not Ionophore induced NFATC1-GFP nuclear import. Moreover, N17 Ras co-transfection does not affect NFATC1-GFP phosphorylation status.

The data presented here clearly suggest that a Rac-1 signalling pathway targets the phosphorylation status of NFATC1 protein in RBL2H3. Possible mechanisms for this effect will be discussed in detail below. The role for Rac-1 in the regulation of NFATC1 sub cellular localisation provides a mechanism for the inhibition of NFAT/AP-1 transcriptional activity observed with N17 Rac-1. NFATC1-GFP nuclear import is not regulated by the Ras GTPase. However, Ras signals are necessary but not sufficient for FcεR1 induction of NFAT/AP-1 transcriptional activity. Given that in T cells there is an established role for Ras in regulation of the AP-1 component of NFAT/AP-1 transcriptional activity a simplistic model can be proposed for the roles of Ras and Rac-1 in regulation of NFAT/AP-1 complexes. Briefly, the data suggest that the FcεR1 regulates the activity of Ras and Rac-1. Parallel signalling pathways may extend from Ras to AP-1 and from Rac-1 to a target controlling the phosphorylation status and hence sub cellular localisation of NFAT. This is a simplistic model for the roles of the Ras and Rac-1 GTPases in the regulation of NFAT/AP-1 transcriptional activity. The data presented below will demonstrate that it is incorrect. In fact the regulation of this transcriptional complex involves two distinct roles for the Rac-1 GTPase.

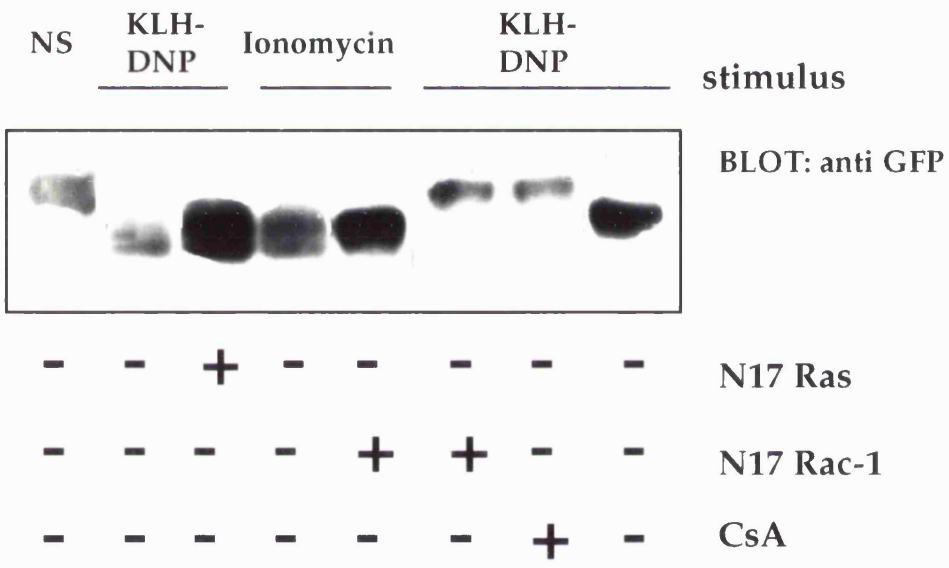


Figure 7.20. FcεR1 mediated NFATC1-GFP dephosphorylation is not inhibited by N17 Ras, Ionomycin mediated NFATC1-GFP dephosphorylation is not inhibited by N17 Rac-1. 10^7 RBL2H3 per point were transfected with 10µg NFATC1-GFP alone or in combination with 20µg pEF N17 Rac-1 or 20µg RSV N17 Ras and recovered for 6h in the absence or presence of 50nM Cyclosporin A (CsA), before either IgE priming and stimulation for 30 min with 500ng/ml KLH-DNP or stimulation with 500ng/ml Ionomycin. Cells were harvested and lysed for Western analysis in a buffer containing 1% NP-40, 50mM HEPES pH7.4, 10mM iodoacetamide, 1mM PMSF and 0.42M NaCl. Proteins were acetone precipitated and resolved by 6% SDS-PAGE before anti-GFP Western analysis using 1µg/ml anti-GFP.

7.2.10 Ras dependent and independent Rac-1 signals target the NFAT/AP-1 transcription factor complex.

In T lymphocytes, NFAT/AP-1 transcription factor complexes control induction of the IL-2 promoter in an antigen receptor dependent manner. The GTPases Ras and Rac-1 are both required for this induction. Whilst no analysis has been made of possible roles for GTPases in the control of NFAT sub cellular localisation in T cells, Ras and Rac-1 are both shown to be important for the regulation of AP-1 dimers in this system. Hence in T cells AP-1 is targeted by two distinct Ras effector pathways; 1) the 'classical' Raf-1/MEK/ERK cascade and 2) a Rac-1 dependent signalling pathway. This is by no means the only example of one GTPase acting as an effector of another. GTPase cascades are described in control of many cellular processes including morphological change and oncogenic transformation. The positioning of Ras and Rac-1 upstream of the NFAT/AP-1 complex in T cells is shown schematically in Figure 7.21.

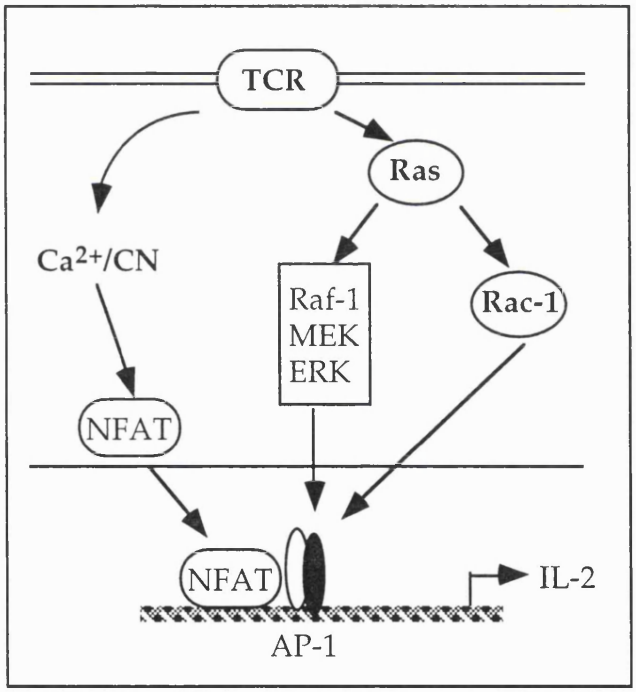
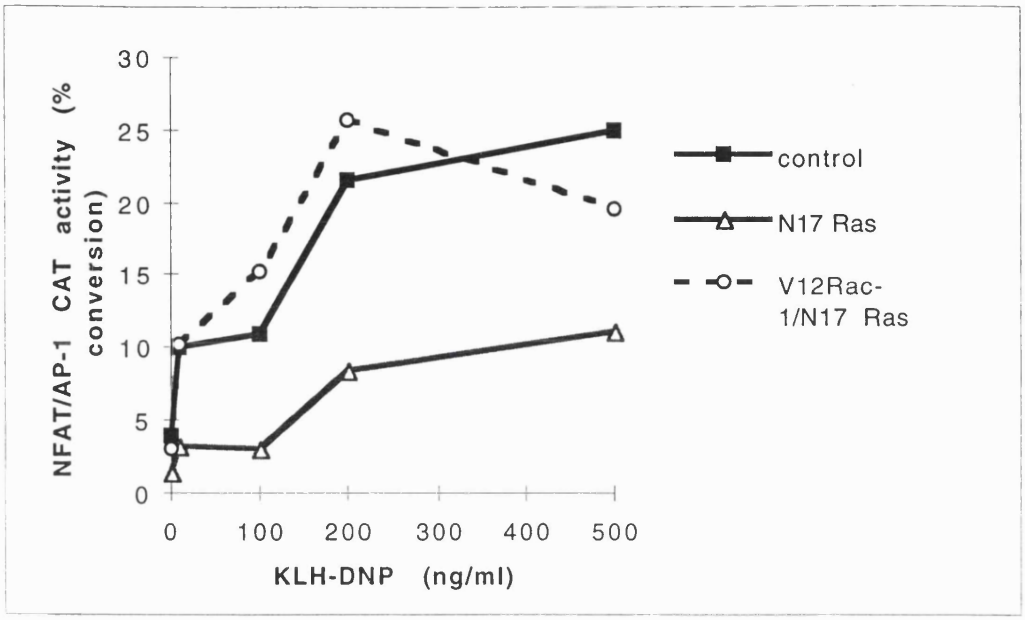


Figure 7.21. Ras and Rac-1 signalling pathways target AP-1 dimer activity in T lymphocytes. Two Ras effector pathways target AP-1 in T cells, via Raf-1/MEK/ERK and the Rac-1 GTPase. TCR, T cell antigen receptor; Ca^{2+}/CN, calcium/calcineurin signals.

A linear Ras/Rac-1 cascade exists in T cells and targets the NFAT/AP-1 complex. In mast cells the Rac-1 dependent nuclear import of NFAT is independent of Ras. The relationship between Ras and Rac-1 in mast cells was examined more closely using the NFAT/AP-1 transcriptional activity assay described in Chapters 5 and 6. In

these experiments rescue of the dominant inhibitory effects of Ras and Rac-1 upon NFAT/AP-1 transcriptional activity was attempted. The data in Figure 7.22a show that N17 Ras cotransfection causes a marked inhibition of NFAT/AP-1 transcriptional activity induced in response to antigenic crosslinking of the FcεR1. However, if N17 Ras and the activated V12 mutant of Rac-1 (V12 Rac-1) are doubly cotransfected, then the inhibition observed with N17 Ras alone is alleviated. Hence V12 Rac-1 rescues N17 Ras inhibition of FcεR1 induced NFAT/AP-1 transcriptional activity. These data would suggest that in fact there is a linear Ras/Rac-1 pathway regulating the activity of the NFAT/AP-1 complex in mast cells. The data in Figure 7.22b show that conversely, an activated mutant of Ras cannot rescue the N17 Rac-1 inhibition of NFAT/AP-1 induction.

a



b

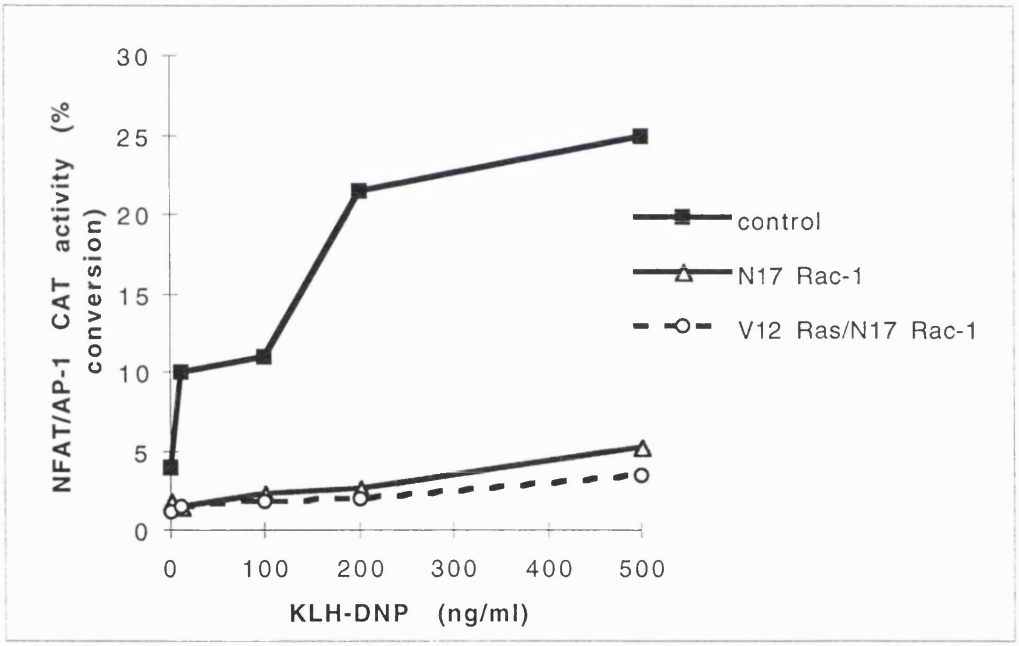


Figure 7.22 Activated Rac-1 can rescue the dominant inhibition of NFAT/AP-transcriptional activity by N17 Ras. 10^7 RBL2H3 per condition were transfected with 15µg IL-4NFAT/AP-1 CAT reporter alone or in combination with the indicated regulatory plasmids (15µg pEF N17Rac-1, 15µg RSV N17 Ras, 15µg pEF V12 Rac-1, 15µg pEF V12 Ras). Cells were recovered for 6h then stimulated using the indicated doses of KLH-DNP for 16h. Cells were harvested and assayed for CAT activity as described. Results are representative of three experiments.

Taken together these observations would place Ras upstream of Rac-1 and the NFAT/AP-1 complex. However, it is equally clear from the assay of NFAT sub cellular localisation that the role of Rac-1 in this process is Ras independent. To reconcile these data a model is proposed in Figure 7.23. Here two pools of Rac-1 are shown to affect distinct processes contributing to NFAT/AP-1 transcriptional activity. First, Rac-1 is postulated to regulate AP-1 activity in a Ras dependent fashion. Secondly, a Ras-independent Rac-1 signal regulated the phosphorylation status and hence sub cellular localisation of NFAT protein.

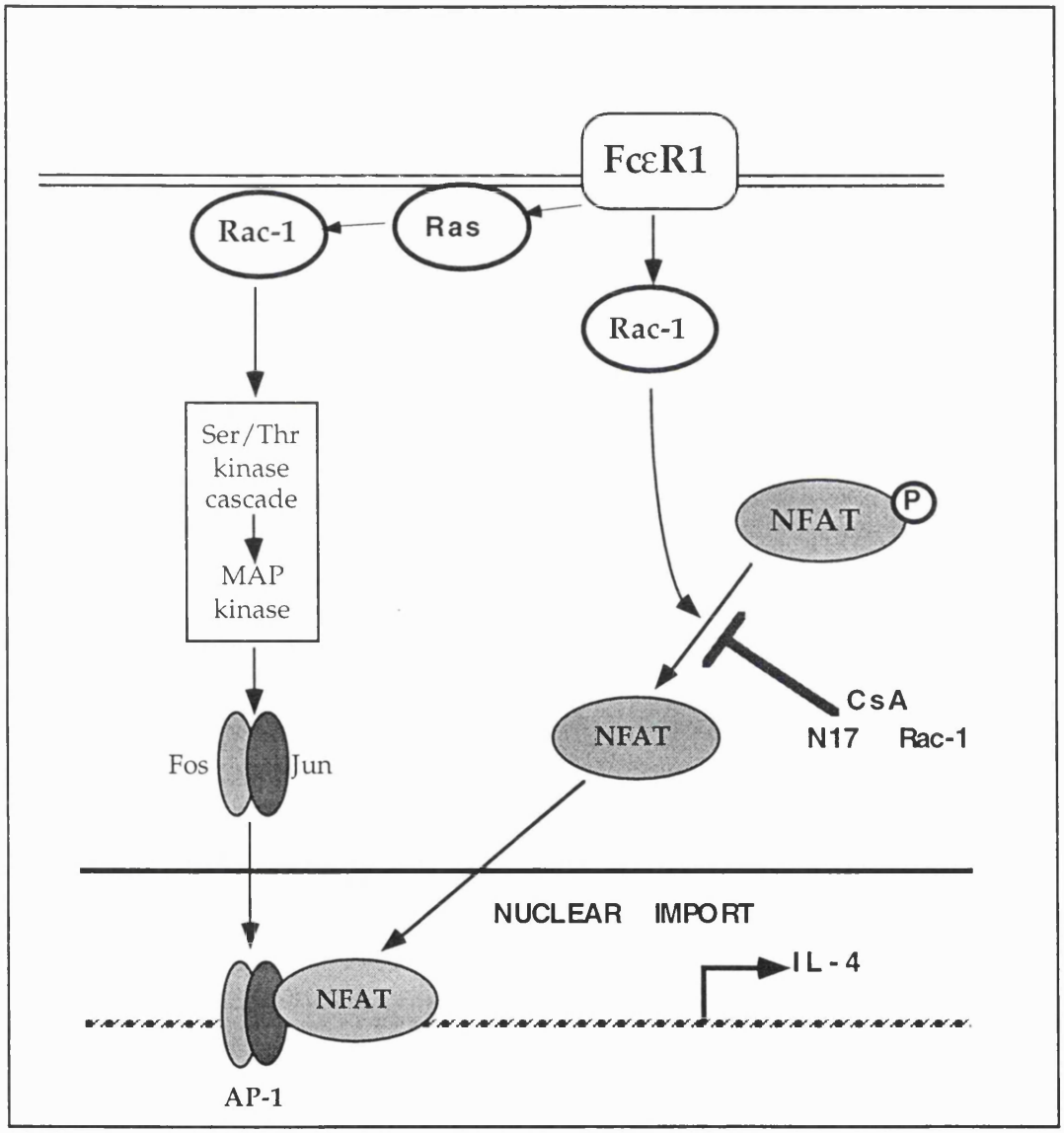


Figure 7.23. Model of NFAT/AP-1 complex regulation by Ras family GTPases in mast cells. Ras and Rac-1 are required for transcriptional activity of the NFAT/AP-1 complex. Ras signals do not regulate the sub cellular localisation of NFAT protein. Rac-1 regulates NFAT phosphorylation status and hence nuclear import. The effect of dominant inhibition of Ras upon NFAT/AP-1 transcriptional activity can be rescued by activated Rac-1, indicating that there is a Ras

dependent Rac-1 signalling pathway acting upon the NFAT/AP-1 complex in addition to the Ras independent effect of Rac-1 upon NFAT sub cellular localisation.

7.3 DISCUSSION

Regulation of cytokine gene promoters by antigen receptors such as the FcεR1 is crucial to the initiation and maintenance of immune responses. NFAT transcription factor complexes control transcriptional induction of numerous genes in mast cells, including those for IL-4, IL-3 and IL-6. The regulation of these complexes requires a combination of signalling events. Production and activation of AP-1 dimers is required, as well as the nuclear import of pre-existing cytosolic NFAT protein molecules. The data presented in Chapter 6 showed that the NFAT/AP-1 transcriptional complex integrates a variety of FcεR1 derived signals; the activity is required of the calcium dependent phosphatase Calcineurin (CN) and two GTPases of the Ras superfamily, Ras and Rac-1. Conventional models developed upon the basis of data from the T lymphocyte system show calcium/CN regulation of NFAT nuclear import, whilst GTPase signals contribute to activation of AP-1. The data presented in this Chapter has detailed studies into the regulation of NFAT sub cellular localisation, with particular reference to the role of Ras family GTPases. The primary finding is that activity of the Rac-1 GTPase is absolutely required for NFATC1 dephosphorylation and nuclear import in the mast cell line RBL2H3. The data extend the current models of NFAT regulation described above and identify a previously unsuspected role for Rac-1.

NFAT transcription factors function in the context of a dimer of AP-1 family proteins. Data obtained using an assay for NFAT/AP-1 transcriptional activity has shown a requirement for these GTPases in both T and Mast cells. Ras and Rac-1 function are clearly required for AP-1 transcriptional activity in the T cell system. Therefore, the role of these GTPases in the induction of NFAT/AP-1 activity has previously been considered to reflect only their contribution to AP-1 activity. The data presented in this Chapter show that Rac-1, but not Ras, directly influences the sub cellular localisation of NFAT protein. Hence the role of Rac-1 in NFAT/AP-1 induction cannot be considered solely in the context of AP-1 regulation. In contrast, there is no evidence from the work presented here that the Ras GTPase functions in any aspect of NFAT/AP-1 regulation other than by signalling to the AP-1 dimer.

7.3.1 Rac-1 is not a regulator of the general nuclear import machinery.

The role for Rac-1 in the control of NFAT sub cellular localisation detailed here raised two possibilities; 1) that Rac-1 selectively controls the nuclear import of NFAT and 2) that Rac-1 is functioning as a component of the general nuclear import machinery. In the context of pathways for the nuclear import of proteins, GTPases are known to be important at the level of the Nuclear Pore Complex (NPC). Here, GTP- and GDP-bound forms of the Ran GTPase are asymmetrically distributed across the nuclear membrane. The active form of Ran is thought to bind and possibly activate the

Importin-β carrier protein. There is evidence that GTPases other than Ran are involved in nuclear import since experiments which render Ran GTP independent show that there is still a GTP requirement for nuclear import to occur. However, the data presented here clearly show that Rac-1 does not function at the level of the nuclear pore complex (NPC). Dominant inhibition of Rac-1 does not cause a general blockade in nuclear import. The NFATC1ΔRel-GFP molecule, which is thought to have a constitutively exposed NLS can enter the nucleus even in the presence of N17 Rac-1. Moreover, delivery of a strong sustained calcium signal using calcium ionophore is able to overcome the N17 Rac-1 block of NFATC1-GFP nuclear import. Therefore it is unlikely that N17 Rac-1 is affecting the NPC.

7.3.2 Rac-1 regulates NFATC1-GFP phosphorylation status.

Dephosphorylation of NFAT molecules is permissive for their subsequent nuclear import. In resting cells NFAT is phosphorylated upon multiple sites within the N-terminal regulatory region. Upon antigen receptor stimulation or exposure to calcium ionophore, dephosphorylation of NFAT is rapid and sustained, reflecting calcium dependent activation of the phosphatase calcineurin. Dephosphorylation is thought to result in a conformational change which exposes previously buried nuclear localisation sequences (NLS). Hence NFAT sub cellular localisation and the transcriptional activity of the NFAT/AP-1 complex is controlled by a balance of regulatory kinase and phosphatase activities. The effect of inhibition of Rac-1 signalling in RBL2H3 is to maintain NFATC1 in a phosphorylated state and hence in a 'closed' conformation where NLS remain buried. Dominant inhibitory Rac-1 mimics the effect of the CN inhibitor CsA which causes cytosolic retention of NFATC1. The mechanism by which Rac-1 modulates NFAT phosphorylation status in this signalling pathway is not clear. Two clear possibilities exist; 1) N17 Rac-1 may enhance the activity of the kinase responsible for NFAT phosphorylation or 2) N17 Rac-1 may inhibit the activity of the NFAT phosphatase, CN.

As described in the Introduction to this Chapter, NFAT is a substrate for one or more kinase activities. Firm data on the identity of the NFAT kinases are not available in antigen receptor stimulated cells. However, it is clear that NFATC1 can be a substrate for Glycogen synthase kinase-3β in Cos and T cells. In a variety of cell types GSK-3β can be negatively regulated in response to activation of protein kinase C or protein kinase B (Akt). If Rac-1 were to be involved in GSK-3β regulation in the FcεR1 system, then negative regulation of PKC or PKB by the FcεR1 would be proposed to be blocked by N17 Rac-1. The net outcome of blockade of this negative regulation would be a positive enhancement of GSK-3β activity. In turn, this would promote NFAT phosphorylation and cytoplasmic retention. There are numerous

qualifications to be noted at this point, not the least of which are that neither PKB or GSK-3β have been shown to be regulated either positively or negatively by the FcεR1.

N17 Rac-1 may inhibit the FcεR1 regulation of the NFAT phosphatase, CN. This possibility supposes a role for endogenous Rac-1 in positive regulation of CN activity. At present, the only reported regulatory signal for CN is a required elevation in levels of intracellular free calcium. Data from the Cos and B cell systems suggests that NFAT activation is dependent on a low level of sustained elevation in [Ca2+]i, rather than a rapid transient increase in [Ca2+]i asscociated with influx from the environment. The former phase of antigen receptor induced calcium flux has been extensively studied and is ascribed to sustained mobilisation of intracellular calcium stores. When the mechanism by which mobilisation of intracellular stores is examined, a hint of a possible role for Rac-1 in this process becomes clear.

Antigen receptors such as the FcεR1 tyrosine phosphorylate and activate Phospholipase Cγ1 (Park *et al.*, 1991a). PLCγ1 hydrolyses its polyphosphoinositide substrate Phosphatidyl inositol 4,5-bisphosphate (PI 4,5-P_2) to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). The latter moiety activates isoforms of Protein kinase C, while the former is a second messenger binds IP3 receptors on the surface of the endoplasmic reticulum and induces the release of calcium from these intracellular stores. This is antagonisistic to the effect of the the sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump responsible for the filling of intracellular calcium stores. In order to sustain this process, it is reasonable that PI 4,5-P_2 levels need to be maintained. This requires that the PI 4-P precursor is phosphorylated by a lipid 5-kinase activity to replenish pools of PI 4,5-P_2. There is a limited amount of data in the literature showing that Rac may be connected to regulation of a phoshatidyl inositol 5' hydroxyl kinase activity. Rac-1 induces PI 4,5-P2 synthesis from PI 4-P in permeabilised platelets (Hartwig *et al.*, 1995). In this study Rac-1 is shown to control a phosphoinositide pathways terminating in the uncapping of filamentous actin. In a second study, Rac-1 was found to associate with PI 5-kinase *in vitro* and *in vivo*. This interaction was independent of the guanine nucleotide binding status of Rac-1 (Tolias *et al.*, 1995). Clearly Rac-1 regulation of PI 5-kinases is not a proven, general phenomenon; it has not been studied in antigen receptor stimulated cells. A possible linkage allows a model such as that in Figure 7.24 to be proposed, where Rac-1 regulation of the PLCγ1 substrate pools connects the GTPase to [Ca^{2+}]i and hence to the activity of CN.

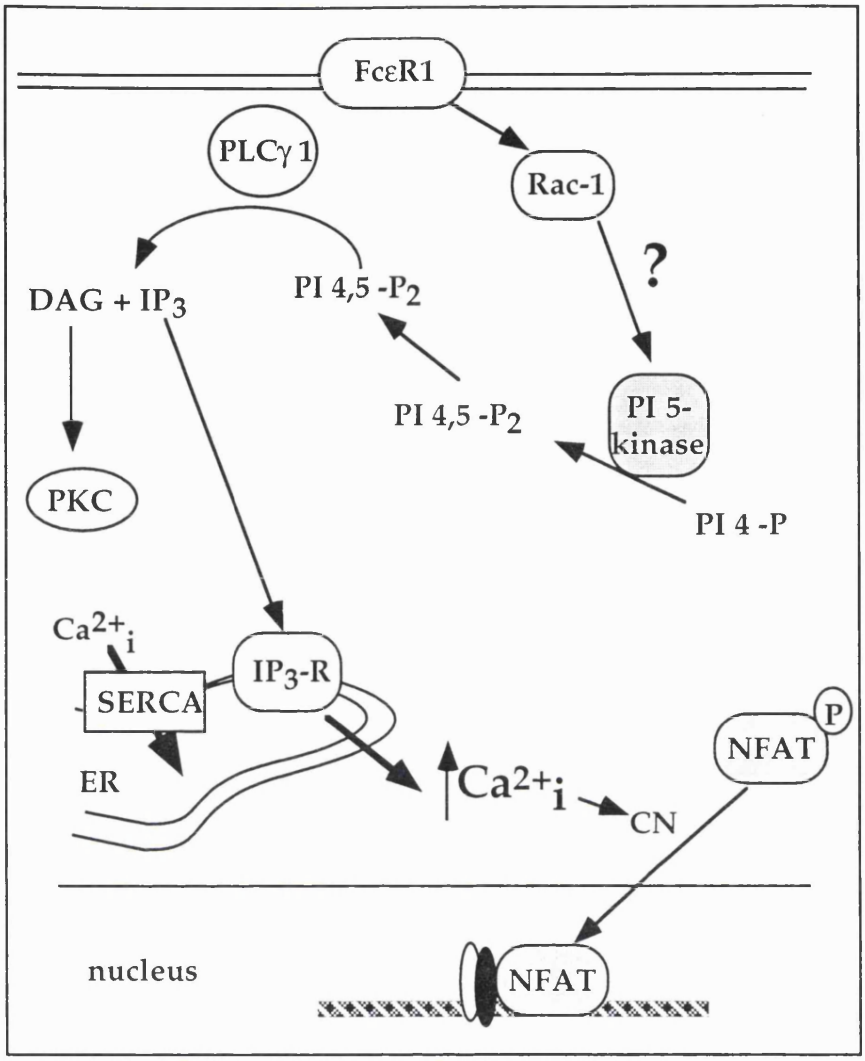


Figure 7.24 Putative Rac-1 regulation of a PI 5-kinase may affect sustained elevations in intracellular free calcium levels. Activity of a PI 5-kinase in mast cells will contribute to provision of a substrate pool for Phospholipase Cγ1 (PLCγ1). PLCγ1-dependent mobilisation of intracellular calcium stores results in activation of the Calcineurin (CN) phosphatase and NFAT dephosphorylation. PKC, protein kinase C; DAG, diacylglycerol; ER, endoplasmic reticulum; PI, phosphatidyl inositol; SERCA, sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase.

7.3.3 Ras dependent and independent Rac-1 signals regulate NFAT/AP-1 activity.

Rac-1 signals which affect NFATC1 nuclear import are independent of the activity of the Ras GTPase. However, Ras signals are necessary for FcεR1 induction of NFAT/AP-1 complex transcriptional activity. A simple model would therefore show two parallel FcεR1 signalling pathways regulating NFAT/AP-1 via Ras family GTPases. In this model, FcεR1 regulation of Ras acts upon AP-1 dimers and a Rac-1 mediated pathway controls NFATC1 sub cellular localisation. This simple model is not adequate to explain the observation that Ras regulates NFAT/AP1 transcriptional

activity in a Rac-1 dependent fashion. These results suggest that in fact Rac-1 has two functions in NFAT regulation. First, Rac-1 is a component of the FcεR1 derived pathways that regulate NFAT nuclear import. Secondly, an FcεR1/Ras mediated pathway regulates the transcriptional activity of nuclear localised NFAT. These data may reflect an important aspect of signal transduction by Ras family GTPases. Functionally distinct pools of a GTPase may co-exist within the same cell and regulate disparate targets in response to similar upstream signals. A model can be suggested for the mechansim by which this functional compartmentalisation may be achieved in that Different Rac-1 effectors may regulate AP-1 and NFAT phosphorylation status.

GTPases of the Ras superfamily regulate diverse cellular responses to a single upstream input by virtue of their multiple effector pathways. Hence, Rac-1 regulation of distinct targets upstream of AP-1 and NFAT phosphorylation status may be accomplished via distinct effector cascades. Yeast two-hybrid screens, genetic analyses, or affinity column purifications have identified a number of putative Rac effectors including the Pak family of serine/threonine kinases and MLK2,3, the tyrosine kinase $p120^{Ack}$, POR1 (partner of Rac1), $p67^{phox}$, Mek kinase (Mekk)-1,4 or phosphatidylinositol 4-phosphate 5-kinase (PI4P 5-kinase). Rac-1 regulation of NFAT sub cellular localisation is not related to Rac-1 effects upon the actin cytoskeleton, hence it is possible to exclude certain effector molecules from a role in the former process. Rac-1 effectors in FcεR1 signalling are not identified. The identification of the relevant Rac-1 effector for control of NFAT sub cellular localisation in the mast cell system is therefore not an immediate prospect.

The Rac-1 GTPase regulates the sub cellular distribution of NFATC1 in mast cells. This role for Rac-1 is unrelated to its regulation of the actin cytoskeleton and does not represent an effect upon the general nuclear import machinery. In fact, the role for Rac-1 lies in the signalling pathways controlling NFATC1 phosphorylation status in the cytoplasm. These data establish a previously unsuspected role for Rac-1 in the complement of antigen receptor driven responses as a regulator of NFATC1 phosphorylation status and sub cellular localisation. As such, Rac-1 is in a position to modulate the transcription of numerous target genes. The GTPase Rac-1 should therefore be considered as a component of the therapeutically important signalling pathways that control NFATC1 nuclear import.

CHAPTER EIGHT

General Discussion

Antigenic crosslinking of the FcεR1 on mast cells results in the expression of inflammatory function. A complex mixture of cytokines, chemokines and allergic mediators is released into the context of an inflammatory site. The interplay between these effector molecules is poorly understood, as are the signalling pathways which control their generation.

Much of the work concerning signal transduction by the FcεR1 has followed a progression similar to that taken by experiments studying the T and B cell antigen receptor systems. Initial experiments, in the case of the FcεR1 examining the control of degranulation, tended to ascribe mast cell activation to a combination of calcium and Protein kinase C signalling events. The major recognised assay for mast cell activation at that time was the measure of allergic mediator secretion. A combination of calcium ionophore and phorbol esters were found to be sufficient to induce secretory responses. Later, by analogy to other antigen receptor systems, it was recognised that multiple protein tyrosine kinases (PTKs) are activated after FcεR1 ligation. The study of mast cell signalling is now in a phase of rapid growth. As for the BCR and TCR, the latest phases have been 1) the identification of numerous receptor-proximal signalling events, 2) the beginning of work placing these into defined signalling cassettes and 3) the identification of functional targets molecules for early signalling proteins, linking the plasma membrane to the cellular compartments at which function is carried out.

At the point that the work in this thesis was started, it was recognised that FcεR1 stimulation resulted in ITAM-dependent activation of the lyn and Syk PTKs, followed by multiple tyrosine phosphorylation events. These had not been placed into defined signalling cassettes, neither had downstream effector molecule targets been identified. Another intriguing aspect of FcεR1 signalling was that no examination of potential regulation or function of Ras family GTPases had been undertaken in this system. The experiments described in this thesis derived from two proposed ideas; 1) that the FcεR1 may regulate adapter molecule cassettes analogous to those which link other antigen receptors to Ras family GTPases and 2) that FcεR1 regulation of Ras family GTPases may control aspects of mast cell function.

The primary observations made in this thesis are summarised below.

1) The FcεR1 regulates components of protein complexes formed around the adapter molecule Grb2. A 33kDa tyrosine phosphoprotein is postulated to be the major adapter between the Grb2 SH2 domain and the FcεR1 ITAMs. The SH3 domains of Grb2 adapt to multiple candidate effector proteins. These include the SLP-76 protein, a newly recognised adapter molecule. By analogy with the T cell system, SLP-76 may link FcεR1/Grb2 to the regulation of the Vav-1 protein. One of the documented functions of Vav-1 is to act as a guanine nucleotide exchange factor for the Rac-1 GTPase. Grb2 SH3 domains also bind the Sos protein, a guanine nucleotide exchange factor for Ras. Protein complexes nucleated around the Grb2 molecule may therefore link the FcεR1 to the regulation of at least two Ras family GTPases.

2) The Ras GTPase regulates distinct nuclear targets of FcεR1 signals by different effector signalling pathways. FcεR1 regulation of transcription factors is a requirement for the induction of multiple genes observed after antigenic crosslinking of the FcεR1 on mast cells. Reporter gene assays were used to assess the contribution of Ras to FcεR1 induction of two transcription factors. Elk-1 regulates the promoter of the immediate early gene c-*fos* as part of a transcriptional complex with the serum response factor. In the RBL2H3 Ras signals are necessary and sufficient for FcεR1 regulation of Elk-1 transcriptional activity. Ras signals to Elk-1 via the Raf-1/MEK/ERK pathway; dominant inhibition of this effector cascade ablates FcεR1 stimulation of Elk-1 transactivation.

Ras is also involved in FcεR1 regulation of the Nuclear Factor of Activated T cells (NFAT) transcription factor. NFAT regulates the promoters of multiple cytokines, growth factors and other immune response genes in a co-operative transcriptional complex with a dimer of AP-1 proteins. FcεR1 induction of Ras is necessary but not sufficient for activation of an NFAT/AP-1 reporter derived from the murine IL-4 promoter. In contrast to Elk-1 regulation, the Raf-1/MEK/ERK effector pathway is not important for FcεR1 regulation of NFAT. Rather, the effect of dominant inhibition of Ras upon NFAT/AP-1 transcriptional activity can be rescued by constitutive activation of the Rac-1 GTPase. These data suggest that regulation of a Rac-1 signalling pathway may be the effector mechanism through which Ras regulates NFAT/AP-1 transcriptional activity.

3) Rac-1 activity is critically important for FcεR1 regulation of NFAT. Reporter assays measuring NFAT transcriptional activity show that Rac-1 function is absolutely required for FcεR1 regulation of this response. The basis of this

requirement for Rac-1 was investigated further. The data showed that Rac-1 regulates the subcellular localisation of NFAT protein in RBL2H3. Dominant inhibition of Rac-1, but not Ras, prevents FcεR1 regulated dephosphorylation and nuclear import of NFAT, providing a mechanism for Rac-1 involvement in regulation of NFAT transcriptional activity. The complexity of this picture was increased by the observation that the effect of dominant inhibition of Ras upon activity of an NFAT/AP-1 reporter gene could be rescued by constitutive activation of the Rac-1 GTPase. This suggests that both Ras dependent and independent Rac-1 signalling pathways contribute to FcεR1 induction of the NFAT/AP-1 transcriptional complex. These are 1) A Ras/Rac-1 pathway postulated to regulate the AP-1 dimer component of the NFAT/AP-1 transcriptional complex and 2) A Ras independent requirement for Rac-1 in FcεR1 regulation of NFAT phosphorylation status and hence subcellular localisation.

The mast cell signalling pathways described here are summarised in Figure 8.1.

At the time the work presented in this thesis was started, there was a partial understanding in the TCR system of the architecture and role of Grb2 adapter molecule complexes. Descriptions of Grb2/Sos complexes in the B cell soon followed. After the initial characterisation of the mast cell Grb2 complexes described here, more functional data on the role of Grb2 'effectors' SLP-76 and Sos has been described in the T cell. The mast cell Grb2 complexes appear functionally and largely structurally similar to those described in the T cell. The degree of conservation between these systems in the structure and function of Grb2 cassettes suggests that they impart benefits of efficiency and sensitivity to early signalling. There are still numerous unanswered questions concerning the adapter molecule function of Grb2 in antigen receptor systems.

First, in T, B and mast cells there are a number of tyrosine phosphoproteins in complex with Grb2 which are unidentified and hence have no assigned function. Second, analyses have tended to concentrate upon proteins which are visibly regulated (i.e. tyrosine phosphorylated) in response to receptor ligation. Proteins which are not PTK substrates may be important uncharacterised players in early signalling via Grb2. Finally, there is the important question of how Grb2 (and other adapters) actually works. How is information passed across the multi-protein complex? It is clear that there is not a simple, linear pathway for the 'upstream' SH2 binding proteins to the SH3 binding 'effectors'. Being part of a receptor regulated Grb2 complex may have at

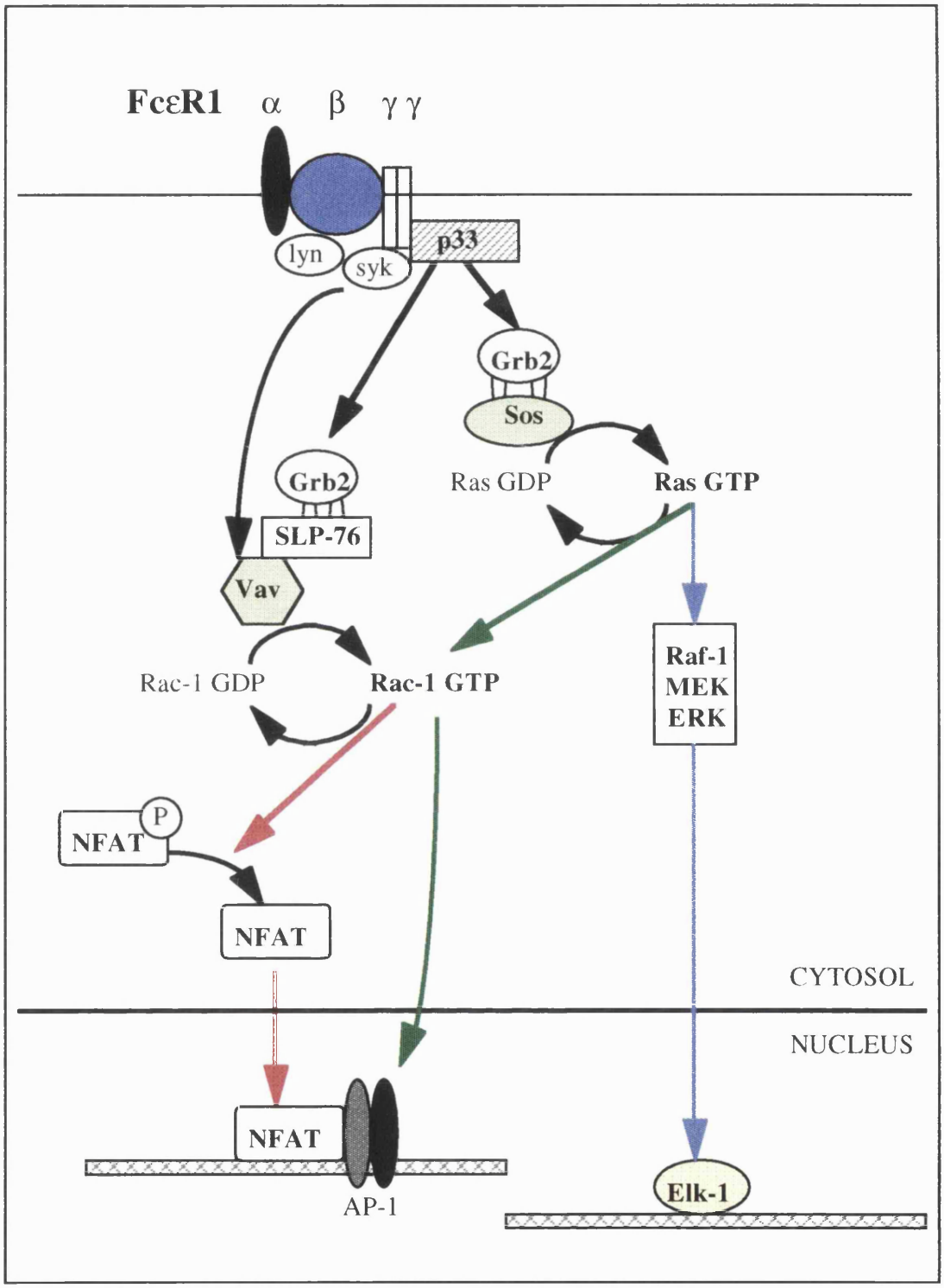


Figure 8.1. FcεR1 signalling pathways described in this thesis. Two Grb2 signalling cassettes are described, which may connect the FceR1 to the regulation of Ras family GTPases. Effector pathways derived from Ras and Rac are shown with the downstream transcription factor targets identified in Chapters 4-7.

least two regulatory effects upon a signalling protein; 1) recruitment into proximity with a substrate, adapter or regulator and 2) binding to Grb2 may alter the conformation of a protein such that it becomes either active or responsive to an upstream activator in its immediate environment.

In the case of the data concerning FcεR1 regulation of Elk-1, a number of notable observations were made. FcεR1 regulation of a transcription factor controlling immediate early gene induction had not previously been described. Neither had there been any report of a downstream target for signals from Ras or any other GTPase in the mast cell system. The mechanism of Elk-1 regulation by Ras closely paralleled that described in other systems including growth factor receptor regulation of the Serum response element in fibroblasts. In contrast, FcεR1 regulation of NFAT/AP-1 transcriptional complexes revealed novel regulatory mechanisms not indicated by experiments in any other cell type.

The regulation of subcellular localisation of NFAT protein is recognised as an important factor in the generation of an immune response. The sheer range of NFAT target genes suggests that the study of mechanisms for regulation of NFAT localisation is likely to result in information upon a process that impinges on many aspects of immunity. Previously calcium/calmodulin regulation of the NFAT phosphatase calcineurin (CN) has been the only upstream signalling pathway described as important in this context. The data presented here have shown that FcεR1 regulation of the Rac-1 GTPase controls NFAT phosphorylation status and hence subcellular localisation. Cyclosporin A and FK506 are clinically highly effective immunosuppressants due to their inhibitory effect upon NFAT dephsophorylation and hence transcriptional activity. The identification of a new player in this process, Rac-1, may have implications for the further study of NFAT subcellular localisation. It is clear that CN is by no means the only target for therapeutic inhibition of NFAT dephosphorylation. It is unlikely that direct inhibition of Rac-1 would be without complications of non-selectivity, a drawback of the current usage of CsA and FK506. However, the burgeoning amount of data upon the numerous Rac-1 effector proteins in other cell systems means that the future possibility of selective suppression by inhibition of a Rac-1 effector molecule cannot be dismissed.

The data presented in this thesis reflects the complexity and efficacy of the manner in which antigen receptors control and use GTPases. The FcεR1 uses two distinct GTPases, Ras and Rac-1 to regulate disparate nuclear targets. Ras, but not Rac-1, regulates Elk-1. Both Ras and Rac-1 regulate NFAT. In the latter case it appears that there may be an even higher level of complexity since Rac-1 is apparently positioned upstream of two different aspects of regulation of the NFAT/AP-1 complex.

The recognition of such complex involvement for GTPases in control of cellular functions inevitably raises the issue of the identity of GTPase effector molecules. It is a characteristic of GTPase signalling that multiple effector proteins adapt an input to multiple downstream targets. In the mast cell system we really have little idea of the effector proteins available to Ras or Rac-1. This situation highlights one of the current limitations of the field of GTPase study. While the number of identified effector proteins for GTPases is increasing, there is as yet no idea of the full complement of effectors available to a GTPase in an antigen receptor regulated cell. Without this type of information, an understanding of the effector mechanisms that target NFAT and Elk-1 cannot be reached. Once effector molecules are identified then the experimental approach of disrupting their function and assaying the consequences is relatively well established. However, these results must always be viewed in the light of the possible cross-talk between effectors.

There are a number of significant questions which merit further study in the mast cell system. The biochemical study of early signalling events in B, T and mast cells has generated a plethora of data on protein-protein interactions. These data can be divided into two categories; 1) those which have been demonstrated to occur *in vivo*, and 2) those that are potential interactions identified using *in vitro* binding assays. It is necessary for a period of critical assessment of these data to take place, where their significance is assayed. In order to do this, a wider complement of functional assays will be required. In the mast cell there are numerous well-defined functional events such as secretion of allergic mediators, cytokine gene induction and morphological change. One of the criteria which could be applied to the current wealth of data upon potential interactions between signalling molecules is that of their subcellular co-localisation. GFP-tagging technology (as described here for assay of NFAT localisation in mast cells) may be applied to some of these issues. There are multiple chromoforms of GFP which permit the localisation of differently tagged proteins to be compared in transfected or microinjected, live, cells. As the resolving limits of confocal analysis increase, it will be possible to closely monitor whether two proteins that are able to interact *in vitro* are actually ever brought into conjunction *in vivo*.

Another important issue in the continuation of mast cell study is that of cellular context. Current assay systems cannot be said to properly model the physiological situation of a mast cell. Mast cells deliver function within the context of a tissue. They are therefore surrounded by other cells, with which they form multiple adhesive interactions, and the numerous protein components of the extracellular matrix. These cannot be viewed as non-signalling interactions. The regulation of intracellular signalling pathways by adhesion molecules is being intensively studied and is not yet fully understood. However, to illustrate, integrin signalling may affect the levels of

intracellular free calcium. This contextual signal will influence the threshold for subsequent antigen receptor regulation of calcium dependent events, such as NFAT dephosphorylation. Exposure to cytokine and other non-antigenic stimuli prior to or concurrently with antigen may also be viewed as a contextual factor in mast cell activation. Hence, to increase the relevance of studies into mast cell signalling, it will be necessary to develop model systems which mimic cellular contexts as well as antigen receptor stimulation.

The study of mast cells is in a period of renaissance. The view that mast cells are an essentially cytotoxic cell type is changing. At one extreme, mast cells have been considered as a reactionary, vestigial, cell type reflecting a human past when parasitic infection was commonplace. In modern Western society the chief notability of the mast cell has been in the phenomenon known as allergy. However, as the data on mast cells becomes more extensive, their position as potential orchestrators of multiple immune responses becomes more clear. They have been found to bear receptors for MHC which are thought to be involved in surveillance for cellular transformation. Mast cells are present in significant numbers at all the surfaces of the body which are repeatedly exposed to environmental antigen such as the gut, lung and skin. The antigenic threshold for mast cell activation at these surfaces may influence commitment to more systemic immune responses than the generation of a local inflammation. Hence, where antigen may determine the threshold for mast cell activation, mast cell activation may in turn determine the threshold for progression of a disease. Conventional approaches exploring mast cell based therapies have focused upon downregulation of mast cell function to combat allergic responses. The therapeutic upregulation of beneficial mast cell function should be considered for the future. An understanding of the signalling processes governing mast cell activation will be fundamental to these applications.

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Regulation of the Adapter Molecule Grb2 by the FcεR1 in the Mast Cell Line RBL2H3*

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Antigenic cross-linking of the high affinity IgE receptor (FcεR1) on mast cells results in protein tyrosine kinase activation. The object of the present study was to explore the regulation of the SH2 and SH3 domain containing adapter molecule Grb2 by FcεR1-stimulated PTK signal transduction pathways. Affinity purification of *in vivo* Grb2 complexes together with *in vitro* experiments with Grb2 glutathione S-transferase fusion proteins were used to analyze Grb2 complexes in the mast cell line RBL2H3. The data show that in RBL2H3 cells several different proteins are complexed to the SH3 domains of Grb2. These include the p21ras guanine nucleotide exchange factor Sos, two basally tyrosine-phosphorylated 110- and 120-kDa molecules, and a 75-kDa protein that is a substrate for FcεR1-activated PTKs. By analogy with Sos, p75, p110 and p120 are candidates for Grb2 effector proteins which suggests that Grb2 may be a pleiotropic adapter. Two Grb2 SH2-binding proteins were also characterized in RBL2H3 cells; the adapter Shc and a 33-kDa molecule. Shc is constitutively tyrosine phosphorylated in unstimulated cells and FcεR1 ligation induces no changes in its phosphorylation or binding to Grb2. In contrast, p33 is a substrate for FcεR1-activated PTKs and binds to Grb2 SH2 domains in FcεR1 activated but not quiescent cells. The β subunit of the FcεR1 is a 33-kDa tyrosine phosphoprotein, but the p33 Grb2-binding protein described in the present report is not the FcεR1 β chain and its identity is unknown. The present report thus demonstrates that there are multiple Grb2 containing protein complexes in mast cells of which a subset are FcεR1-regulated. Two other of the Grb2-binding proteins described herein are tyrosine phosphorylated in response to FcεR1 ligation: the 75-kDa protein which binds to Grb2 SH3 domains and the 33-kDa protein that associates with the Grb2 SH2 domain. We propose that protein complex formation by Grb2 is an important consequence of FcεR1 cross-linking and that this may be a signal transduction pathway which acts synergistically with calcium/PKC signals to bring about optimal mast cell end function.

Antigenic cross-linking of the high affinity immunoglobulin E (IgE) receptor (FcεR1) on mast cells and basophils results in expression of the production of cytokines and exocytotic secretion of allergic mediators (1). The stimulation of PTKs[1] by the activated FcεR1 is an immediate membrane proximal event crucial for FcεR1 signal transduction and hence mast cell end function (2–4). The FcεR1 complex is composed of three subunits, the 45-kDa α and 30-kDa β chains and a homodimer of two disulfide-linked 10-kDa γ chains (5). The intracellular tails of the β and γ subunits contain a common motif, termed the tyrosine-based activation motif (TAM) of the general sequence $EX_2YX_2L/IX_7YX_2L/I$,(6) which is thought to couple the FcεR1 to intracellular PTKs. TAMs are found also in the invariant subunits of the B cell and T cell antigen receptors (BCR and TCR) and thus appear to be an evolutionarily conserved sequence motif essential for the activation of lymphocytes (7, 8–11).

Members of the TAM-based receptor family are typically coupled to two subfamilies of PTKs. The FcεR1 associates with the src-family tyrosine kinase p56lyn and a 72-kDa PTK, Syk (3, 12). The BCR is similarily associated with p56lyn(13) and Syk whereas the TCR is predominantly associated with the src-family p59fyn and a kinase homologous to Syk, ZAP70 (14, 15). It has also been reported that FcεR1 cross-linking induces tyrosine phosphorylation and activation of the atypical src-like Bruton tyrosine kinase (16) suggesting that a third category of PTKs may contribute to FcεR1 signaling. One common response to triggering of the FcεR1, TCR, and BCR is tyrosine phosphorylation and activation of phospholipase C-γ1 (2, 17, 18). This permits these receptors to control inositol polyphosphate and diacylglycerol production which in turn modulate intracellular calcium concentration and the activation of PKC, respectively. Calcium flux and PKC play a critical role in TCR signal transduction (19, 20) and are important signals for mast cell activation and the secretion of granule components such as histamine and 5-hydroxytryptamine (21, 22).

A second, essential, PTK-controlled signaling pathway to originate from the TCR involves the guanine nucleotide-binding proteins p21ras (23). The mechanism of TCR coupling to p21ras was proposed to involve the guanine nucleotide exchange protein Sos, the homologue of the *Drosophila* "son of sevenless" gene product (24, 25). Sos is known to complex with the adapter protein Grb2/Sem 5 which is composed of one SH2 domain and two SH3 domains (26, 27). In many cell systems, the SH3 domains of Grb2 bind to Sos whereas the interaction between the Grb2 SH2 domain and tyrosine-phosphorylated molecules such as the epidermal growth factor (EGF) receptor or Shc may regulate the function and cell localization of Sos (27–30).

Studies in T lymphocytes have identifed a number of ty-

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[1] The abbreviations used are: PTKs, protein tyrosine kinases; TAM, tyrosine-based activation motif; BCR, B cell antigen receptor; TCR, T cell antigen receptor; EGF, epidermal growth factor; GST, glutathione S-transferase; DNP, dinitrophenol; KLH, keyhole-limpet-hemocyanin; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

rosine-phosphorylated proteins which can potentially bind to Grb2. These include Shc, a membrane-localized tyrosine phosphoprotein of 36-kDa and 75- and 116-kDa molecules (25, 31–34). The 75- and 116-kDa molecules are constitutively associated with the SH3 domains of Grb2 analogous to the Grb2/Sos association and are substrates for TCR-activated PTKs (32, 33). The p75 is apparently a hematopoietic cell-specific protein. Its structure and protein sequence are not known, but on the basis of its association with the Grb2 SH3 domains, p75 is a candidate for a second Grb2 downstream effector molecule. Shc and p36 when tyrosine phosphorylated bind to the SH2 domain of Grb2. In TCR-activated T cells, both Shc and p36 are tyrosine phosphorylated and could potentially form a complex with Grb2 SH2 domains. However, the predominant complexes that can be detected are composed of p36-Grb2-Sos (25, 35). Studies in B cells have generated evidence for regulation of Grb2 complexes by the BCR. In this system both Shc tyrosine phosphorylation and the formation of Shc-Grb2-Sos complexes are observed in response to BCR ligation (36, 37) The pattern of PTK activation in response to BCR or FcεR1 triggering is similar, particularly in the stimulation of the 72-kDa PTK Syk (12). However, there has been no analysis of the regulation of adapter molecules such as Grb2 and Shc in FcεR1-stimulated cells. Accordingly, the object of the present studies was to examine whether Grb2 and associated molecules are regulated by the FcεR1.

The data presented here show that the SH3 domain of Grb2 binds Sos and 75-, 120-, and 140-kDa tyrosine phosphoproteins whereas the major Grb2 SH2 domain-binding proteins are Shc and an unknown 33-kDa protein. Two of the Grb2-binding proteins described herein are tyrosine phosphorylated in response to FcεR1 ligation: the 75-kDa protein which binds to Grb2 SH3 domains and the 33-kDa protein that associates with the Grb2 SH2 domain. Shc is apparently not a substrate for FcεR1-activated PTKs and hence its association with Grb2 is not controlled by cell activation. The present report thus demonstrates that there are multiple Grb2-containing protein complexes in mast cells of which a subset are FcεR1-regulated.

MATERIALS AND METHODS

Antibodies—The anti-phosphotyrosine monoclonal antibody 4G10 was purchased from Upstate Biotechnology Inc (Lake Placid, NY). The anti-phosphotyrosine antibody FB2 (W. Fantl, University of California) was purified from hybridoma supernatant. Polyclonal anti-Shc antibody was purchased from TCS Biologicals. Monoclonal anti-Dinitrophenyl (mouse IgE isotype) was purchased from Sigma. Anti-mouse Sos1 and anti-Grb2 were purchased from Upstate Biotechnology Inc. and Affiniti (Nottingham, United Kingdom), respectively. The monoclonal antibody JRK1, with specificity for the β chain of FcεR1 (38), was a generous gift of Dr. Juan Rivera (NIAMS, Bethesda, MD).

Peptide Reagents and Fusion Proteins—The EGFR-Y1068 peptide sequence is PVPEpYINQS. The Trk-Y490 peptide sequence is IENPQpYFSDA. For use in affinity purification these peptides were coupled to Affi-Gel 10 beads (Bio-Rad). The following glutathione *S*-transferase (GST) fusions were used and have been previously described (28, 32). GST-alone, GST-Grb2 (full-length Grb2), GST-μSH3 49L/203R double SH3 mutant, GST-Grb2NSH3 isolated amino-terminal SH3 domain (amino acids 1–58), GST-Grb2 CSH3 isolated carboxyl-terminal SH3 domain (amino acids 159–217) and GST-Sos (mSos1 carboxyl-terminal residues 1135–1336). The fusion proteins were coupled to glutathione-agarose beads (Sigma) for affinity purification experiments.

Cell Culture—The rat basophilic leukemia cell line RBL2H3 was a gift of Dr. Roberto Solari, Glaxo Research and Development, U.K. They were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated (56 °C for 30 min) fetal bovine serum and 2 mM glutamine. Only the adherent fraction of the cell cultures was passaged or used in experiments.

Cell Stimulation and Lysis—RBL2H3 monolayer cells were detached from the culture flask using a cell scraper, washed once, and primed in suspension with 1 μg/ml IgE anti-dinitrophenol (IgE anti-DNP) in RPMI 1640, 10% fetal bovine serum for 1 h at 37 °C. Receptor cross-linking was effected using 10 μg/ml keyhole limpet hemocyanin (KLH)-DNP conjugate (Calbiochem) at 37 °C. After stimulation all cells were placed on ice and pelleted immediately in a microfuge. The cells were lysed in a buffer containing 50 mM HEPES (pH 7.4), 1% (w/v) Nonidet P-40, 150 mM NaCl, 20 mM NaF, 20 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml protease inhibitors (leupeptin, pepstatin A, and chymotrypsin), and 1 mM Na_3VO_4.

Affinity Purifications and Western Blotting—Lysates (1.5×10^7 RBL2H3) were precleared with Protein A-insoluble suspension (Sigma) for 15 min at 4 °C. Lysates for fusion protein affinity purifications were then precleared with glutathione-agarose bead suspension (Sigma). Lysates for peptide precipitations (Trk-Y490 and EGFR-Y1068) were cleared with Affi-Gel 10 (Pharmacia). Affinity purifications with specific reagents were carried out for 2 h at 4 °C with constant rotation. The beads were washed three times in 1 ml of lysis buffer and boiled in reducing SDS sample buffer for 10 min. Samples were resolved on 11% SDS-PAGE.

The resolved proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) which was blocked in 5% non-fat milk for 1 h. The membrane was probed with a specific antibody followed by an appropriate second stage horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham). Reactive bands were detected using the enhanced chemiluminescence system (Amersham).

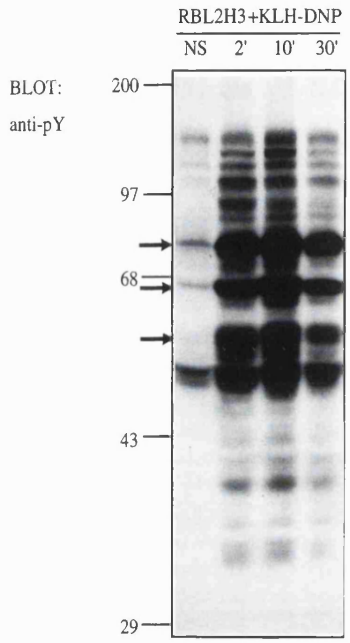

FIG. 1. **Anti-phosphotyrosine Western blot of total cell lysate from RBL2H3.** RBL2H3 were primed with 1 μg/ml IgE anti-DNP for 1 h at 37 °C before stimulation by cross-linking of the receptor with KLH-DNP at 10 μg/ml for the time indicated in minutes. *NS* refers to non-stimulated cells. Acetone precipitations of post-nuclear protein from Nonidet P-40 lysates were resolved under reducing conditions on 11% SDS-PAGE and transferred to PVDF before blocking with non-fat milk. Each track contains cell lysate from 10^6 cells. The membrane was then probed with 1 μg/ml of the anti-phosphotyrosine antibody 4G10 for 1 h and developed with anti-mouse Ig-horseradish peroxidase.

RESULTS

Tyrosine Phosphorylation of Multiple Grb2 Binding Proteins Is an Immediate Consequence of FcεR1 Cross-linking—The data in Fig. 1 show a Western blot analysis with anti-phosphotyrosine antibodies of total cell lysates from quiescent and FcεR1-stimulated RBL2H3 cells. The experiment demonstrates that there is prominent FcεR1-induced tyrosine phosphorylation of proteins at 80, 70, and 55 kDa. This phosphorylation is rapid, detected within 2 min and sustained for a period of 30 min. In control experiments no increase in tyrosine phosphorylation of any proteins in RBL2H3 cells which have been incubated with monomeric IgE but not exposed to cross-linking multivalent antigen was observed. Binding experiments with a GST-fusion protein of wild type Grb2 were carried out in order to establish whether FcεR1-induced tyrosine phos-

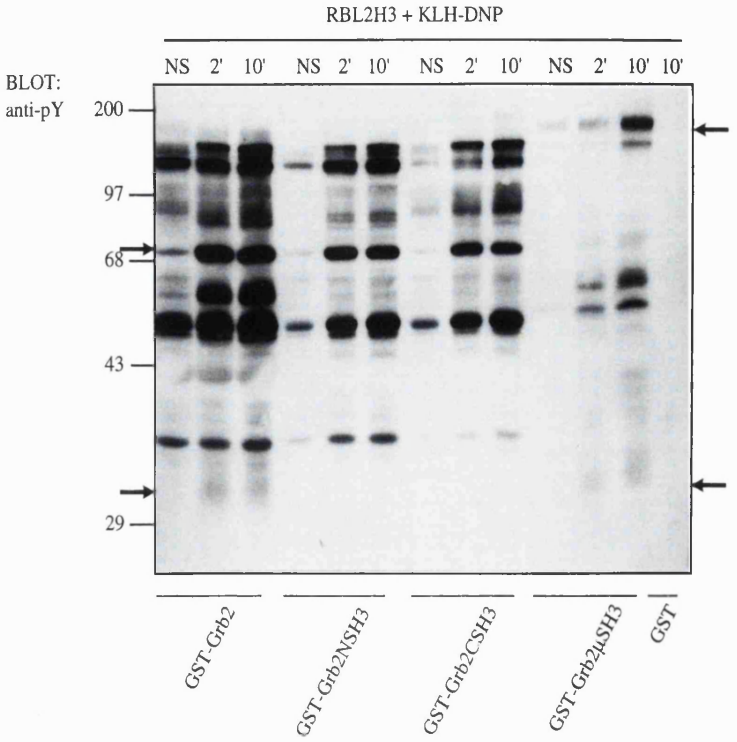

FIG. 2. **Anti-phosphotyrosine Western blot showing SH domain specificity of tyrosyl proteins binding to Grb2 in RBL2H3 cells.** 2×10^7 RBL2H3 cells/point were primed as described and stimulated with 10 μg/ml KLH-DNP for the time indicated in minutes at 37 °C. Lysates were affinity purified for 2 h with the Grb2 fusion protein indicated. The panel of GST fusion proteins used were the wild type GST-Grb2, the single SH3 domain truncations GST-Grb2NSH3 and GST-Grb2CSH3, and the double SH3 mutant GST-Grb2 μSH3. GST alone coupled to glutathione-agarose beads was included as a negative control. Samples were resolved on 11% SDS-PAGE under reducing conditions before transfer to PVDF membrane, blocking with non-fat milk, and Western blot analysis with 4G10.

phoproteins include a subset of Grb2-binding proteins. The data from this experiment are shown in Fig. 2. In precipitates purified from RBL2H3 cell lysates with GST-Grb2, there are multiple tyrosine phosphoproteins in both quiescent and activated cells. Many proteins are observed which are basally tyrosine phosphorylated in unstimulated cells. However, there is an FcεR1-induced increase in tyrosine phosphorylation of proteins migrating at 120, 110, 75, 65, and 30–33 kDa.

The experiment in Fig. 2 explores also the SH domain specificity of FcεR1-induced tyrosine-phosphorylated Grb2-binding proteins. Affinity binding experiments were performed with truncated or mutated Grb2 fusion proteins comprising the isolated Grb2 NH_2-terminal and COOH-terminal SH3 domains, called GST-Grb2NSH3 and GST-Grb2CSH3, respectively. In addition, GST-Grb2 with mutations in both SH3 domains which leave the SH2 domain intact (GST-Grb2 μSH3) was used. Fig. 2 shows a Western blot analysis with anti-phosphotyrosine antibodies on proteins affinity purified from RBL2H3 lysates using the panel of GST-Grb2 variants in comparison with wild-type GST-Grb2. The data show that a prominent FcεR1-induced tyrosine phosphoprotein of 75 kDa was detected in the precipitates isolated with wild type GST-Grb2, GST-Grb2NSH3, or GST-Grb2CSH3 fusion proteins. Both GST-Grb2 SH3 domain fusion proteins also purified tyrosine-phosphorylated proteins of 38, 52–55, 120, and 140 kDa. Cross-linking of the FcεR1 induced tyrosine phosphorylation of 33- and 65-kDa proteins which bound to the wild type GST-Grb2 fusion protein. These species did not bind the isolated Grb2 SH3 domains. In addition to the isolated SH3 domains, further characterization of domain specificities was possible using the GST-Grb2 μSH3 molecule. This is a full-length Grb2 construct which has point mutations introduced into each of the SH3 domains but an intact SH2 domain. The FcεR1-induced 75-kDa tyrosine phosphoprotein did not bind to the GSTGrb2 μSH3 fusion protein, nor did the 110-kDa tyrosine phosphoprotein. However, FcεR1-induced tyrosine phosphoproteins of 33 and 65 kDa bound efficiently to the GST-Grb2 μSH3 protein. As well, a 55-kDa tyrosine phosphoprotein and a doublet of high molecular mass tyrosine-phosphorylated proteins at 120–140 kDa were detected binding to GST-Grb2 μSH3. The latter species were inducibly tyrosine phosphorylated in FcεR1-activated RBL2H3 to varying degrees between experiments.

From these binding studies it can be concluded that there are FcεR1-induced tyrosine phosphoproteins of 33 and 65 kDa which can bind to Grb2 SH2 domains. In addition, there is an FcεR1-induced tyrosine phosphoprotein of 75 kDa which binds to Grb2 SH3 domains. Other tyrosine phosphoproteins that associate with Grb2 SH3 domains and SH2 domains have molecular masses of 110–120 and 120–140 kDa, respectively. The binding experiments using truncated and mutated Grb2 fusion proteins show that the proteins migrating in the 110–120 kDa range may in fact be resolved into both basally and FcεR1-induced tyrosine-phosphorylated proteins. Similarly, there are multiple tyrosine-phosphorylated proteins at 52–55 kDa binding to the intact GST-Grb2 fusion protein that resolve into both basally phosphorylated proteins and FcεR1-induced tyrosine phosphoproteins upon analysis with the truncated and mutant Grb2 fusion proteins.

Tyrosine Phosphoproteins That Associate with the SH3 Domains of Endogenous Grb2 in RBL2H3 Cells—Initial experiments showed that there is a complicated pattern of FcεR1-induced tyrosine phosphoproteins which are capable of binding to Grb2 fusion proteins. Therefore, to establish whether there are FcεR1-induced complexes between tyrosine-phosphorylated proteins and endogenous Grb2, we used affinity purification protocols to isolate Grb2 and associated proteins from RBL2H3 cell lysates. This technique has been described in detail previously (32) and has been used effectively to examine Grb2 complexes in TCR-activated T-lymphocytes. Briefly, a synthetic tyrosine phosphopeptide corresponding to the autophosphorylation site Tyr-1068 in the carboxyl-terminal tail of the epidermal growth factor receptor (EGFR-Y1068) was used to precipitate Grb2 from RBL2H3 cells. The EGFR-Y1068 peptide binds to the Grb2 SH2 domain with nanomolar affinity and thus competitively blocks associations between cellular proteins and the SH2 domains of Grb2. Use of this peptide allows the copurification of Grb2 and proteins complexed with Grb2 SH3 domains. Grb2 was also purified from RBL2H3 cells using a GST fusion protein of a proline-rich fragment from the carboxyl terminus of murine Sos (GST-Sos). The GST-Sos fusion

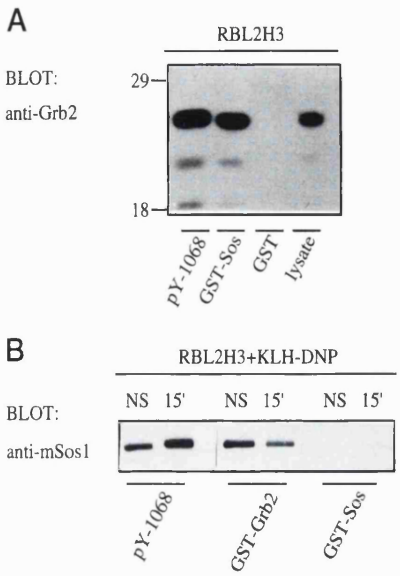

FIG. 3. **The EGFR-Y1068 peptide and GST-Sos fusion protein affinity purify Grb2 from RBL2H3 cell lysate.** RBL2H3 cell lysates containing 2×10^7 cell equivalents were precleared as described and affinity purified with either EGFR-Y1068 coupled to Affi-Gel-10 or GST-Sos fusion protein coupled to glutathione-agarose beads. Postnuclear proteins were acetone precipitated from 2×10^6 RBL2H3 (total lysate). The samples were resolved on 11% SDS-PAGE under reducing conditions before transfer to PVDF and blocking of the membrane. *A*, the membrane was then probed with 0.5 μg/ml anti-Grb2 for 1 h and developed with anti-mouse Ig-horseradish peroxidase second stage antibody. *B*, the PVDF membrane was blocked and probed with 1 μg/ml anti-mSos1 for 2 h before development with anti-rabbit Ig-horseradish peroxidase second stage antibody.

protein used in these experiments binds the Grb2 SH3 domain and therefore isolates Grb2 and proteins associated with the Grb2 SH2 domain. GST-Sos competitively prevents the copurification of Grb2 SH3-binding proteins. The data in Fig. 3 show an anti-Grb2 Western blot of the cellular proteins purified with GST-Sos or EGFR-Y1068 and indicate that these two reagents isolate equivalent levels of endogenous Grb2. Moreover, a comparison of the levels of Grb2 purified with EGFR-Y1068 or GST-Sos with Grb2 levels in total cell lysates (Fig. 3*A*) shows that these reagents are capable of affinity purifying the majority of endogenous Grb2 from RBL2H3 cells. The data in Fig. 3*B* show that it is possible to copurify Sos with Grb2 when the EGFR-Y1068 motif but not GST-Sos is used as an affinity matrix.

To determine whether any of the tyrosine phosphoproteins observed in GST-Grb2 affinity purifications bind to endogenous Grb2 via its SH3 domains, we performed Western blot analysis with phosphotyrosine antibodies on Grb2 complexes isolated with EGFR-Y1068 peptide. As shown in Fig. 4*A*, the EGFR-Y1068 peptide precipitates a 75-kDa tyrosine phosphoprotein from FcεR1-activated but not quiescent RBL2H3 cells. The EGFR-Y1068 peptide also purified two tyrosine phosphoproteins of 110 and 120 kDa which were apparently equally phosphorylated in quiescent and FcεR1-activated cells. These tyrosine phosphoproteins appear to bind to endogenous Grb2 via its SH3 domains. They do not coprecipitate with Grb2 which has been isolated using the GST-Sos fusion protein to compete out Grb2 SH3-binding proteins (Fig. 4*B*). They also comigrate with the 75-, 110-, and 120-kDa tyrosine phosphoproteins detected in the binding experiments using GST-Grb2 single SH3 domain fusion proteins (Fig. 2). Affinity purification with the GST-Grb2 fusion protein produces the characteristic pattern of tyrosine phosphoproteins observed in previous experiments. It should be noted that there was a variable nonspecific binding of a 50–52-kDa doublet of tyrosine phosphoproteins to control

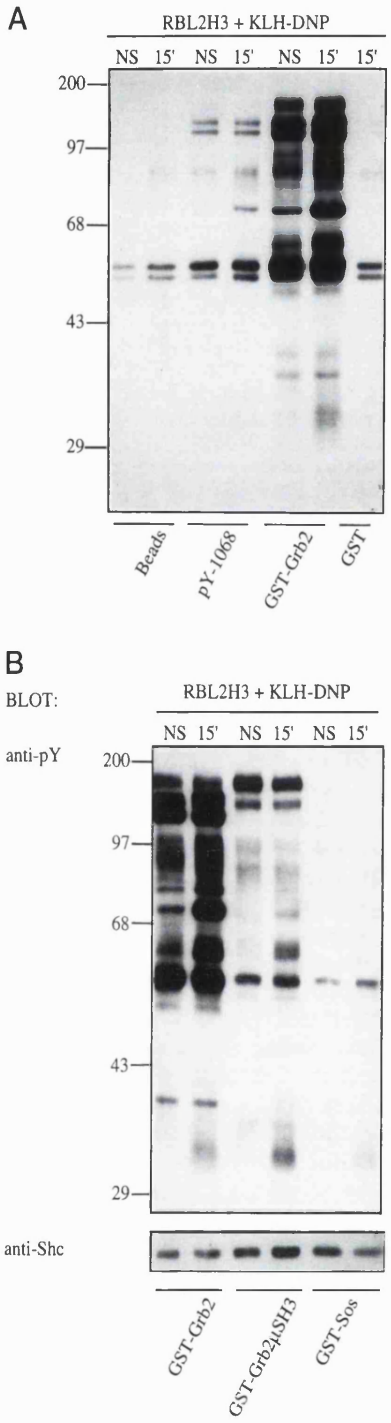

FIG. 4. *A*, the EGFR-Y1068 phosphopeptide purifies tyrosine phosphoproteins from stimulated RBL2H3. RBL2H3 were primed with IgE anti-DNP and stimulated with KLH-DNP for 15 min as described. Lysates were made, and affinity purifications were carried out on 2×10^7 cell equivalents/lane with the following reagents: Affi-Gel-10 beads alone, EGFR-Y1068 coupled to Affi-Gel-10, GST-Grb2 fusion protein, and GST alone coupled to glutathione-agarose beads. The samples were resolved under reducing conditions on 11% SDS-PAGE before transfer and blocking as described above. The membranes were then probed with 1 μg/ml 4G10 for 1 h and developed with anti-mouse Ig-horseradish peroxidase. *B*, binding of 55- and 33-kDa tyrosine phosphoproteins to endogenous Grb2 in RBL2H3. RBL2H3 cells were stimulated and lysed as described previously. Lysates from 2×10^7 cells/point were precleared and affinity purified for 2 h with either GST-Grb2, GST-Grb2 μSH3, or GST-Sos. After 11% SDS-PAGE and transfer to PVDF membrane, Western blot analysis was carried out with 1 μg/ml 4G10. The membrane was then stripped and reprobed with 1 μg/ml anti-Shc overnight at 4 °C and developed with anti-rabbit Ig-horseradish peroxidase.

affinity purifications comprising GST alone or Affi-Gel beads alone.

Tyrosine Phosphoproteins of 33 and 55 kDa Associate with Endogenous Grb2 SH2 Domains—The data in Fig. 4*B* show anti-phosphotyrosine Western blots of the endogenous Grb2 complexes isolated from RBL2H3 cells with GST-Sos. These data indicate that the 120-, 140-, and 65-kDa tyrosine phosphoproteins observed binding to the normal and the double SH3 mutant GST-Grb2 fusion proteins do not coprecipitate with endogenous Grb2. However, an FcεR1-induced tyrosine phosphoprotein of 33 kDa was detected in the endogenous Grb2 complexes purified by GST-Sos. In addition, a tyrosine phosphoprotein of 55 kDa was observed to bind to endogenous Grb2 in both quiescent and FcεR1-stimulated RBL2H3. These p33 and p55 molecules appeared to bind to Grb2 SH2 domains based on their pattern of binding to the panel of Grb2 fusion proteins. As well, they did not coprecipitate with the Grb2 complexes purified with the EGFR-Y1068 peptide, and their interaction with endogenous Grb2 was competed by the EGFR-Y1068 peptide that binds to the Grb2 SH2 domain (data not shown).

In many cells the adapter molecule Shc is tyrosine phosphorylated in response to receptor stimulation and forms a complex with Grb2 SH2 domains. Western blot analysis with Shc antisera has shown that RBL2H3 cells express two isoforms of Shc. The 55-kDa isoform is predominant and the 46-kDa Shc protein is a minor component of the Shc population in these cells. The data in Fig. 4*B* show that a tyrosine phosphoprotein of 55 kDa is coprecipitated with endogenous Grb2 by the GST-Sos fusion protein from both quiescent and FcεR1-stimulated RBL2H3 cells. This protein had a slightly lower mobility than the nonspecific 50–52-kDa proteins described earlier and could thus possibly represent tyrosine phosphorylated 55-kDa Shc isoform. Accordingly, the blots were reprobed with a Shc antiserum. Fig. 4*B* shows that the 55-kDa Shc isoform binds to the wild type and SH3-mutated Grb2 fusion proteins and also can be copurified with endogenous Grb2 by GST-Sos. The binding of Shc to endogenous Grb2 was not influenced by the activation state of the RBL2H3.

To further analyze the tyrosine phosphorylation of Shc in RBL2H3 cells, we used a tyrosine phosphopeptide Trk-Y490, which corresponds to a high affinity binding site for Shc SH2 domains in the Trk subunit of the nerve growth factor receptor. This was used as a reagent to affinity purify Shc from RBL2H3 cells. The data in Fig. 5*A* show an anti-Shc Western blot of proteins affinity purified with the Trk-Y490 peptide from RBL2H3 cells. The 55- and 46-kDa Shc isoforms bind to the Trk-Y490 peptide but not to the EGFR-Y1068 peptide used here as a specificity control. The data in Fig. 5*B* show an anti-phosphotyrosine Western blot of Trk-Y490 peptide precipitates isolated from quiescent and FcεR1-triggered RBL2H3 cells. The 55-kDa Shc isoform is tyrosine phosphorylated in both quiescent and stimulated cells. These data demonstrate also that the 55-kDa tyrosine phosphoprotein binding to GST-Grb2 and GST-Sos comigrates with the tyrosine-phosphorylated 55-kDa Shc protein purified with Trk-Y490. The nonspecific 50–52-kDa protein doublet can once again be observed binding to control Affi-Gel beads alone but with a different mobilty to either of the Shc isoforms.

The 33-kDa Grb2 SH2 Domain-binding Tyrosine Phosphoprotein Is Not the β Subunit of the FcεR1—The accumulated evidence from the experiments in Figs. 1–5 show that FcεR1 triggering results in tyrosine phosphorylation of a 33-kDa protein which binds to endogenous Grb2 in an SH2 domain-mediated association. The β subunit of the FcεR1 is 30–33 kDa and is known to be tyrosine phosphorylated in response to receptor

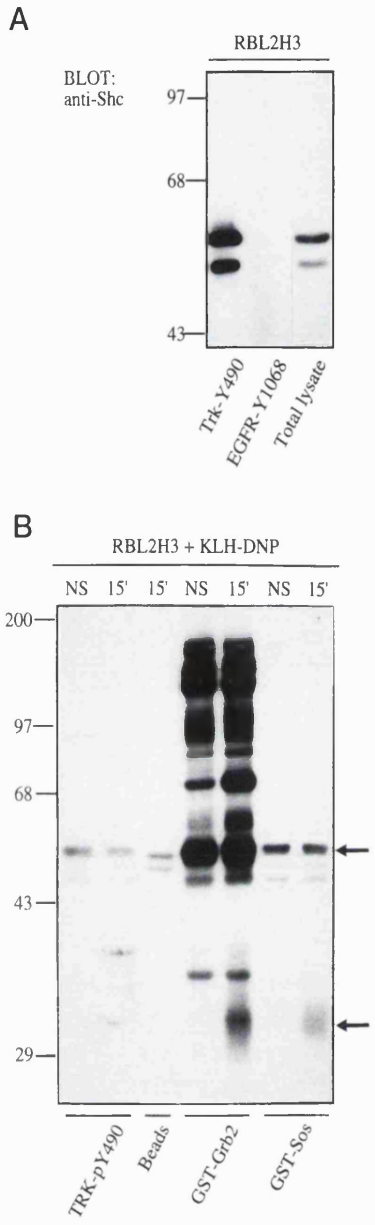

FIG. 5. **Shc is basally tyrosine phosphorylated in RBL2H3 mast cells.** *A*, RBL2H3 were lysed and affinity purifications were then carried out with Affi-Gel-coupled Trk-Y490 or EGFR-Y1068 peptide as indicated on the figure, using 3×10^7 cell equivalents/point. Acetone precipitation of post-nuclear protein from 3×10^6 cells was carried out. All samples were then resolved on 11% SDS-PAGE under reducing conditions and transferred to PVDF for probing with specific antibody. Western analysis was carried out using anti-Shc at 1 μg/ml overnight at 4 °C prior to development with anti-rabbit Ig-horseradish peroxidase. *B*, RBL2H3 cells were FcεR1 stimulated as described. Affinity purification of precleared lysates was carried out for 2 h using Trk-Y490 coupled to Affi-Gel-10 beads or the GST fusion proteins indicated. As a negative control Affi-Gel-10 beads alone were used to purify RBL2H3 lysates. The samples were resolved and transferred to PVDF as described. Western blot analysis was carried out using 1 μg/ml 4G10.

triggering. Accordingly, we examined whether the p33 Grb2-binding protein was the β subunit of the FcεR1 (Fig. 6). Western blot analysis with the FcεR1 β chain antibody JRK1 showed that the β chain can be immunoprecipitated with the anti-phosphotyrosine antibody FB2 from FcεR1-stimulated but not quiescent cells (Fig. 6). Furthermore, it was possible to observe a hyperphosphorylated population of JRK1-immunoreactive β chain molecules in total lysates from FcεR1-activated RBL2H3. This latter population comigrated on SDS-PAGE with the population selected for by the FB2 anti-phosphoty-

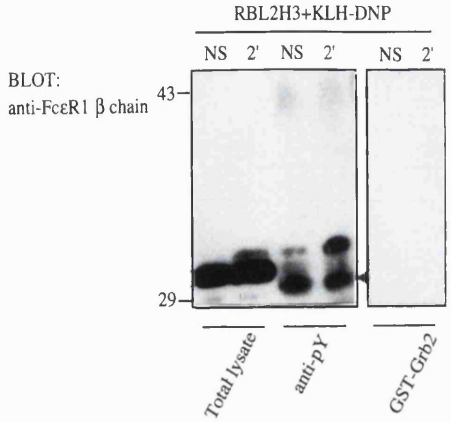

FIG. 6. **The p33 tyrosine phosphoprotein binding the Grb2 SH2 domain in RBL2H3 cells is not the FcεR1 β chain.** Affinity purifications of lysates from 2×10^7 quiescent or FcεR1-stimulated RBL2H3 were carried out using GST-Grb2. Post-nuclear protein from 2×10^6 stimulated or unstimulated RBL2H3 cells was acetone-precipitated. Immunoprecipitations using 2 μg/point of the anti-phosphotyrosine antibody FB2 were carried out on lysates from 2×10^7 quiescent or FcεR1-stimulated RBL2H3. The samples were resolved and transferred as described. The membrane was probed with the mouse monoclonal antibody JRK1, which is reactive for the FcεR1 β chain, overnight at 4 °C. The Western blot was then developed with an anti-mouse Ig-horseradish peroxidase second stage antibody. An *arrow* marks the position of antibody light chain in the anti-phosphotyrosine immunoprecipitations.

rosine immunoprecipitation. However, the 33-kDa Grb2-binding tyrosine phosphoprotein detected by anti-phosphotyrosine Western blot analysis of GST-Grb2 affinity purifications from FceR1-stimulated RBL2H3 was not immunoreactive with the JRK1 antibody and is thus not the β subunit of the FcεR1. It is apparent, moreover, that the SDS-PAGE mobility of p33 is different from the tyrosine-phosphorylated β chain.

DISCUSSION

The data presented here show that in RBL2H3 mast cells, as in many cell systems, the guanine nucleotide exchange factor for p21ras, Sos, is complexed to the SH3 domains of Grb2. The data show also that there are FcεR1-induced complexes between tyrosine phosphoproteins and the adapter molecule Grb2. These include two basally tyrosine-phosphorylated 110- and 120-kDa molecules and a 75-kDa protein substrate for FcεR1-activated PTKs which binds to the Grb2 SH3 domain. Two protein substrates for FcεR1-activated tyrosine kinases were observed to bind GST fusion proteins of Grb2 but more importantly were also found in association with endogenous Grb2. A 75-kDa protein which was tyrosine phosphorylated in activated but not quiescent RBL2H3 cells was associated with the SH3 domains of Grb2. We also detected a 33-kDa tyrosine phosphoprotein complexed to Grb2 SH2 domains in FcεR1 stimulated but not quiescent cells. Previous studies of Grb2-binding proteins in T cells have illustrated how experiments that identify Grb2-binding proteins solely on the basis of affinity purifications with GST-Grb2 fusion proteins can be misleading. This point is made again by the present study. Hence wild type GST-Grb2 fusion protein experiments suggested that in RBL2H3 lysates there are tyrosine phosphoproteins of 33, 55, 65, 120, and 140 kDa which are capable of binding to Grb2 SH2 domains. The 33- and 65-kDa proteins in particular are substrates for FcεR1-activated PTKs as evidenced by their inducible tyrosine phosphorylation. However, only the constitutively tyrosine-phosphorylated 55-kDa and the FcεR1-induced 33-kDa tyrosine phosphoproteins were detected in association with endogenous Grb2 SH2 domains. Similarly, only a subset of the tyrosine-phosphorylated proteins identified as Grb2 SH3-binding proteins by the *in vitro* binding experiments were present in the *in vivo* Grb2 complexes isolated from RBL2H3 cells: a 75-kDa FcεR1-induced tyrosine phosphoprotein and two proteins of 110 and 120 kDa, respectively, which were equivalently tyrosine phosphorylated in quiescent and activated cells and appeared to associate constitutively with Grb2 SH3 domains.

It has been described in many cell systems that tyrosine-phosphorylated Shc forms a complex with Grb2 (28, 39). In the present study, Shc was observed to be tyrosine phosphorylated in quiescent RBL2H3 cells, and no increases in Shc tyrosine phosphorylation were detected in response to FcεR1 triggering. This suggests that Shc is not a major substrate for FcεR1-activated tyrosine kinases in RBL2H3 cells. In accordance with the lack of FcεR1 control of Shc tyrosine phosphorylation, no FcεR1-induced changes in Shc-Grb2 complex formation were observed in RBL2H3 cells although Shc was constitutively associated with Grb2 in an SH2 domain-mediated association. It has been previously noted that there is no significant formation of Shc-Grb2 complexes in response to TCR ligation in T cells (25, 35). In contrast, in B cells ligation of the BCR induces tyrosine phosphorylation of Shc and induces a Shc-Grb2-Sos complex that is proposed to link the BCR to p21ras (36, 37). Interestingly, Shc is a reasonably abundant protein in both T cells and mast cells. Moreover, in both cell types cytokines such as interleukin-2 and interleukin-3 can stimulate high levels of Shc tyrosine phosphorylation and induce Shc-Grb2 complexes indicating that there are no technical problems in detecting such complexes in these cells (40–42).

In the absence of inducible tyrosine phosphorylation of Shc, a possible candidate for linking signals transduced by the FcεR1 to the Grb2 signaling pathway is a 33-kDa tyrosine phosphoprotein. This p33 is tyrosine phosphorylated in response to FcεR1 stimulation and binds Grb2 SH2 domains. The β subunit of the FcεR1 has a similar molecular weight to p33 and is tyrosine phosphorylated in response to receptor ligation (17). We have shown, however, that the p33 tyrosine phosphoprotein observed to bind the SH2 domain of endogenous Grb2 is not the β chain of the FcεR1. This does not exclude an indirect interaction between Grb2 and the FcεR1. The FcεR1-induced p33 molecule appears functionally analogous to the TCR-induced p36 phosphoprotein observed as the major tyrosine-phosphorylated species to bind Grb2 SH2 domains in activated T cells (25, 35). It remains to be determined, however, whether p36 and p33 share any structural homology. In this context, studies in a variety of cell systems have identified many tyrosine kinase substrates with apparently diverse functions which can bind to Grb2 SH2 domains. These include adapters such as Shc, tyrosine phosphatases such as R-PTPα and Syp, and growth factor receptors and receptor subunits such as the EGF receptor and IRS-1 (27, 43, 44). Accordingly, it appears that multiple mechanisms have evolved to couple cellular PTKs to Grb2, of which the p36 and now p33 molecules may be one type.

One well-documented role of Grb2 is to couple tyrosine kinases to the guanine nucleotide exchange protein Sos and hence to p21ras signaling pathways (45). We have observed that triggering of the FcεR1 in RBL2H3 cells results in activation of p21ras and downstream effector molecules such as Raf-1 and the MAP kinase ERK2.[2] The current data demonstrate that Sos is constitutively associated with Grb2 SH3 domains in RBL2H3 cells. Accordingly, the FcεR1-induced 33-kDa tyrosine phosphoprotein that binds to Grb2 SH2 domain may be an adapter that couples the FcεR1 to Grb2-Sos complexes thereby permitting FcεR1 stimulation of p21ras. However, it has been recognized increasingly that Grb2 may link PTKs to molecules

[2] H. Turner and D. Cantrell, unpublished observations.

other than Sos. For example, a second p21ras guanine nucleotide exchange protein C3G binds to Grb2 SH3 domains in PC12 cells (46). As well, the GTPase dynamin can bind to and be activated by Grb2 SH3 domains *in vitro* and *in vivo* (47). In the present report, we have shown that in RBL2H3 cells Grb2 SH3 domains do not only bind the p21ras guanine nucleotide exchange protein Sos. There are also tyrosine-phosphorylated proteins of 75, 110, and 120 kDa which associate with endogenous Grb2 SH3 domains. By analogy with Sos, C3G, and dynamin, the p75, p110, and p120 molecules are candidates for alternate Grb2 effector molecules. The p75 molecule is of particular interest in this context because it is a substrate for FcεR1-activated PTKs. One of the major substrates for FcεR1-activated PTKs has been recently cloned and is a 75-kDa protein termed SPY or HS1, which has an SH3 domain and also contains proline-rich sequences reminiscent of SH3-binding domains characterized in molecules such as Sos (48). Western blot analysis of endogenous Grb2 complexes in both T cells and RBL2H3 cells demonstrated that HS1 is not the Grb2-associated p75 described in this study.[3] Associations between Grb2 and p75 have not been detected in fibroblasts suggesting that this molecule has a limited tissue distribution and hence function (32). In preliminary analysis the FcεR1-induced p75 appears identical to a 75-kDa protein that is a substrate for TCR-activated PTKs and is constitutively associated with Grb2 SH3 domains in T cells (32). We propose that p75 may be a common signaling element for the TAM-based receptor family that includes the TCR and the FcεR1. Recently, $p120^{cbl}$, which is a substrate for TCR-activated PTKs, was shown to bind to the NH_2-terminal SH3 domain of Grb2 fusion proteins (34). The constitutively tyrosine-phosphorylated 120 kDa protein described herein to associate with endogenous Grb2 complexes was not $p120^{cbl}$ although, p120 ^{cbl} was detected in the Grb2 fusion protein complexes isolated from RBL2H3 cells and corresponded to the 120-kDa FcεR1-induced tyrosine phosphoprotein seen in these complexes (data not shown).

The role of the FcεR1 is to regulate the exocytosis of mast cell granules and to modulate cytokine gene expression. The present data have shown that the FcεR1 induces tyrosine phosphorylation of multiple Grb2-binding proteins, strongly suggesting that this adapter protein is involved in coupling the FcεR1 to one or more signal transduction pathways. It has been demonstrated that calcium and PKC have an important role in FcεR1-regulated cellular responses (21). We would propose that Grb2-coupled signaling pathways may also be important for FcεR1-induced mast cell activation.

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Distinct Ras Effector Pathways Are Involved in FcεR1 Regulation of the Transcriptional Activity of Elk-1 and NFAT in Mast Cells

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Summary

Activation of Ras GTPases is a conserved feature of antigen receptor signaling, including FcεR1 activation of mast cells. Antigenic cross-linking of the FcεR1 on mast cells results in secretion of allergic mediators and induction of immediate early and cytokine genes. Here we examine the role of Ras in coupling the FcεR1 to transcriptional regulation. The transcription factors Elk-1, an immediate early gene regulator and the nuclear factor of activated T cells (NFAT), in the context of the IL-4 gene, are identified as Ras targets in mast cells. Ras mediates diverse effects via its diverse effector pathways, which may include other members of the Ras GTPase family such as RhoA and Rac-1. We observe that Elk-1 and NFAT are targeted by distinct Ras effector pathways in mast cells. Activation of the "classical" Ras/Raf-1/MEK/ERK cascade is necessary and sufficient for FcεR1 induction of Elk-1. Ras function is required, but not sufficient for FcεR1 induction of NFAT. However, activation or inhibition of Ras markedly shifts the antigen dose-response for FcεR1 induction of NFAT. The effector pathway for Ras activation of NFAT is not Raf-1/MEK. We identify that the Rac-1 GTPase is critical in FcεR1 regulation of NFAT, acting either in parallel with or as an effector of Ras. These data place Ras in a crucial position in mast cells, regulating disparate nuclear targets. Moreover, we identify that two GTPases, Ras and Rac-1, are important regulators of NFAT, and therefore of cytokine expression in mast cells.

Antigenic cross-linking of the high affinity receptor for IgE (FcεR1) on mast cells results in expression of inflammatory function (1). In addition to the release of inflammatory mediators from cytoplasmic granules, there is a significant nuclear component to the activation of mast cells after FcεR1 ligation. Expression of various genes is induced, notably leading to de novo synthesis of cytokines such as IL-4, -6, GM-CSF, and TNFα (2).

The FcεR1 complex is tetrameric, comprised of 45-kD α and 30-kD β chains and a homodimer of two disulfide-linked 10-kD γ chains (3). Antigenic cross-linking of receptor-bound IgE results in aggregation of FcεR1 complexes in the plane of the plasma membrane and rapid activation of cytoplasmic protein tyrosine kinases (PTKs)[1] (4). Immunoreceptor tyrosine-based activation motifs present in FcεR1 β and γ couple the receptor to the src family PTK p56lyn and to p72syk (3). FcεR1-associated PTKs activate a number of effector pathways which together control mast cell function. These include PLCγ1 activation and subsequent generation of inositol polyphosphate and diacylglycerol second messengers (5, 6). These, in turn, modulate intracellular Ca^{2+} levels and protein kinase C (PKC) activation, respectively. There are defined roles for Ca^{2+} and PKC signals in FcεR1 regulation of exocytosis (7, 8), and a Ca^{2+}/calcineurin dependent pathway is known to be important in the induction of a number of cytokine genes (2, 9).

Our previous work has shown that in addition to Ca^{2+}/PKC signals, FcεR1-regulated PTKs are coupled to effector pathways via the adaptor molecule Grb2 (10). Grb2 forms protein complexes through interactions of its SH2 and SH3 domains with molecules which may be substrates for receptor-associated PTKs (11). One Grb2 effector molecule in the mast cell is Sos, the mammalian homologue of the *Drosophila* "Son of Sevenless" protein (10). Sos is a guanine nucleotide exchange factor that promotes GTP-loading, and hence activation of the GTPase Ras and its effector pathways. Studies in various systems have placed Ras in a critical position regulating diverse cellular processes through its regulation of kinase cascades with transcription factor targets.

[1] *Abbreviations used in this paper:* CAT, chloramphenicol acetyl transferase; CN, calcineurin; CsA, cyclosporin A; dn, dominant inhibitory; MAP, mitogen-activated protein; MEK, Erk-activating kinase; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; PTK, protein tyrosine kinase.

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It is recognized that Ras is able to activate multiple effector signaling pathways. An effector pathway mediated by the Raf-1 serine/threonine kinase has been extensively characterized in numerous systems (12). Raf-1 is recruited to the plasma membrane by active Ras.GTP. This recruitment results in Raf-1 activation and subsequent activation of the mitogen-activated protein (MAP) kinases Erk1 and Erk2 by the Erk-activating kinases (MEKs). Like the B and T cell antigen receptors, the FcεR1 activates the Ras/Raf-1/Erk cascade, but its importance in antigen receptor signaling is not clear. Moreover, studies of the role of Ras in regulating fibroblast transformation have concluded that the Raf-1/MEK pathway does not mediate all Ras effector functions (13). Similarly, in T lymphocytes, the Raf-1/MEK pathway has been shown to mediate Ras effects on positive selection of thymocytes but apparently is not required for Ras control of T cell proliferative responses (14). Alternative effectors for Ras are less defined than the "classical" Raf-1/MEK/Erk cascade, but include the Ras GTPase activating proteins (Ras-GAPs) (15), PtdIns-3′ hydroxyl kinase (16), and GDS proteins for the GTPase Ral (17). There is also a consensus that Ras responses are coupled to signaling networks mediated by Rho family GTPases such as Rac-1, CDC42, and RhoA (18, 19). The role of these Rho-family GTPases in antigen receptor responses is not well characterized, but there is an increasing awareness from both biochemical and genetic analyses that these molecules will have important functions in the immune system. Vav, which is one of the immediate substrates for PTKs associated with antigen receptor complexes (20), has a domain homologous to Dbl, a guanine nucleotide exchange factor for Rho family proteins (21). Recent studies have shown that Vav can induce Rac-1–mediated activation of the MAP kinase JNK-1 (22). In a clinical context, the Wiskott-Aldrich Syndrome immunodeficiency has been mapped to a defect in expression of an effector protein for the GTPase CDC42 (23).

Previous studies have shown that FcεR1 induction of a number of cytokine genes is sensitive to the imunosuppressive drug cyclosporin A (CsA) (24). The CsA sensitivity of cytokine gene induction reflects the importance of the CsA target molecule, the calcium dependent phosphatase calcineurin (CN), in this process. CN is critical for the regulation of the nuclear factor of activated T cells (NFAT), a transcription factor involved in the regulation of various cytokine genes (25, 26). In addition to the role of calcium/CN, a previous report has shown that Ras signals link the FcεR1 to regulation of the IL-5 cytokine gene (27). However, there is no description of Ras effector pathways involved in coupling the FcεR1 to nuclear events, or in determining the repertoire of such targets for Ras signals. The present report identifies the transcription factors Elk-1 and NFAT as targets for Ras signals in mast cells. Moreover, we explore the Ras effector pathways used to link the FcεR1 to these targets. The data show that the FcεR1 regulates Elk-1 transactivation via a Ras/MEK-dependent pathway. The FcεR1 activation of Elk-1, described herein for FcεR1 signaling, provides the paradigm for involvement of the "classical" Ras/Raf-1/MEK cascade in coupling antigen receptors to gene transcription. We also show that NFAT in the context of the IL-4 promoter is another nuclear target for Ras signals downstream of the FcεR1. However, in contrast to Elk-1, FcεR1 regulation of NFAT is accomplished by an effector pathway for Ras distinct from Raf-1/MEK. The present study also provides the first evidence that a member of the Rho family of GTPases, Rac-1, can regulate FcεR1 induction of NFAT. These data reveal the complexity and efficacy of Ras and Rho family GTPases in coupling antigen receptors to diverse nuclear events.

Materials and Methods

Reagents. KLH-DNP conjugate, ionomycin calcium salt, CsA and phorbol-12,13 dibutyrate were from Calbiochem (La Jolla, CA). Monoclonal IgE anti-DNP was from Sigma Chemical Co. (St. Louis, MO), as were all reagents for chloramphenicol acetyl transferase (CAT) assays except ^{14}C acetyl coenzyme A from Amersham International (Buckinghamshire, England). MEK inhibitor PD095089 was from New England Biolabs (Beverly, MA). The PKC inhibitor Ro-318425 was a gift from Dr. David Williams (Roche Pharmaceuticals, Welwyn, UK). Anti-pan–Erk antibody was from Affiniti (UK).

Cell Culture and Stimulation. The rat basophilic leukemia cell line RBL2H3 as maintained in DMEM supplemented with 10% (vol/vol) heat-inactivated (56°C for 30 min) fetal bovine serum. Only the adherent fraction of the cell cultures was passaged or used in experiments. Cells were detached from culture substrate using cell scrapers, washed once, and primed with 1 μg/ml IgE anti-DNP in DMEM, 10% fetal bovine serum for 2 h at 37°C. Receptor cross-linking was effected using 500 ng/ml KLH-DNP conjugate (Calbiochem) at 37°C. Other stimuli were applied as follows: 50 ng/ml PdBu, 500 ng/ml ionomycin.

Western Blotting. Conditions for RBL2H3 lysis and Western blotting were as described previously (10). Briefly, postnuclear lysates were prepared, and cellular proteins were acetone precipitated and then resolved by 15% SDS-PAGE. Proteins were transferred to polyvinyl difluoride membrane which was blocked in 5% nonfat milk for 1 h at room temperature before probing with 1 μg/ml anti-Erk (Affiniti). The membrane was washed three times in PBS/0.02% Tween-20, and incubated with 0.5 μg/ml goat anti–mouse horseradish peroxidase conjugate. After washing as above, Erk bands were visualized by enhanced chemiluminescence (Amersham International).

Plasmids. LexA OP.tk CAT comprises two copies of the LexA reporter. In conjunction with the pEF-NLexA Elk-1C fusion protein, its use has already been described (28). This system involves the transfection of a reporter gene construct of a LexA binding site upstream of the CAT reporter gene under a thymidine kinase minimal promoter (LexA OP.tk CAT). A plasmid encoding a fusion protein of the LexA DNA binding domain and Elk-1 COOH-terminal region under a constitutive promoter is cotransfected (pEF-NLexA Elk-1C). The LexA region of the fusion protein binds constitutively to its site on the reporter gene construct. Activation signals phosphorylate the Elk-1C region of the fusion protein, leading to recruitment of transcriptional machinery and CAT gene induction. The IL-4 NFAT CAT reporter was a gift of E. Serfling (Wurzburg University, Wurzburg, Germany) and comprised a trimerized NFAT/AP-1 site derived from purine box B of the murine IL-4 promoter (GATCCTGAGTTTACATTGGAAAATTTTATAGAGCGAGTTG). Plasmids encoding

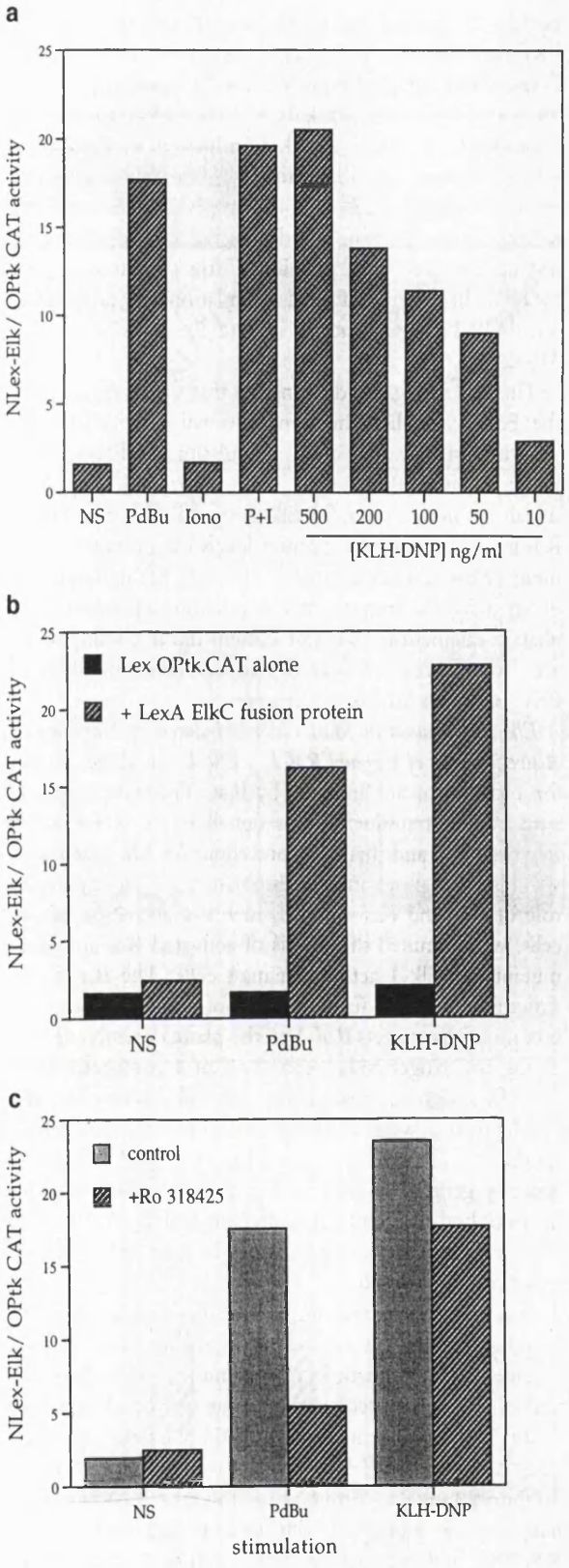

Figure 1. (*a*) FcεR1 activation stimulates Elk-1 transactivation in mast cells. 12 μg LexA OPtk.CAT and 6 μg LexA Elk-1C plasmids were used per 1 × 10^7 cells in electroporation at 310 V/960 μF. Cells were recovered as described in Materials and Methods before stimulation for 16 h with PdBu (50 ng/ml), Ionomycin (500 ng/ml) alone or in combination (*P*+I), or IgE priming followed by antigenic cross-linking of the FcεR1 with the indicated dose of KLH-DNP. Control cells (*NS*) were left unstimulated. (*b*) Stimulation of LexA OP.tk CAT activity is dependent on the presence of the LexA Elk-1C fusion protein. 1 × 10^7 cells per stimulus were electroporated as described with 12 μg LexA OPtk.CAT alone or in combination with 6 μg LexA Elk-1C. Cells were recovered, and then stimulated for 16 h as described above. (*c*) FcεR1 activation of Elk-1 is not PKC-dependent. Cells were transfected with the Elk-1 reporter system as described, and recovered for 6 h. Preincubation with either 1 μM Ro-318425 or DMSO carrier was 30 min at 37°C before stimulation with either PdBu (50 ng/ml) or KLH-DNP (250 ng/ml).

signaling proteins were as follows: pEF-BOSv-HaRas (constitutively active V12Ras), RSVN17Ras (dominant inhibitory Ras), and pEF-dnRaf-1 (dominant inhibitory Raf-1, Raf-1 residues 1-257). The active pEF-V12Rac, dominant inhibitory pEF-N17Rac, and active pEF-V14Rho mutants have all been previously described (29). The constitutively active membrane targeted pEF-Raf-1CAAX was a gift of J. Downward (Imperial Cancer Research Fund). Plasmid DNA was prepared by CsCl density gradient centrifugation.

Transient Transfection Assays Using CAT Reporter Gene Constructs. Transient transfection was carried out using a Beckman Gene-Pulser electroporation apparatus. RBL2H3 monolayer cells were detached from the culture flask using a cell scraper, and resuspended at 2 × 10^7 cells/0.6 ml DMEM + 10% FCS at 37°C. Cell were pulsed in 0.4 cm cuvettes (Beckman) at 310 V, 960 μF before pooling, dilution in complete medium, and division at 1 ml/well between wells of a 24-well tissue culture plate. Cells were allowed to recover for 6 h at 37°C before priming and stimulation as indicated.

For CAT reporter gene assays 5 × 10^6 cells were lysed in 150 μl buffer containing 0.65% (vol/vol) NP-40, 10 mM Tris, pH 8.0, 1 mM EDTA, and 150 mM NaCl for 15 min on ice. Lysates were then transferred to a 68°C water bath for 10 min. Cell debris was pelleted, and aliquots of lysate were removed to a fresh tube in an assay volume of 100 μl to which 40 μl of a start solution containing 0.5 mM acetyl CoA, 5 mM chloramphenicol, 0.5 M Tris, pH 8.0, and 1 μl/point of 50 μCi/ml ^{14}C acetyl coenzyme A was added. The assay was incubated for 16 h at 37°C before chloramphenicol was extracted using ethyl acetate. The amount of radioactivity in the acetylated product and nonacetylated substrate for each reaction was determined by liquid scintillation counting of organic and aqueous phases, respectively. Results are expressed as percentage conversion of chloramphenicol to the acetylated form.

Results

Elk-1 Transactivation Is Induced by the FcεR1 and Phorbol Esters, But Not by the Action of Calcium Ionophore. The effects of various stimuli on Elk-1 activity in mast cells were assessed using a CAT reporter gene assay for Elk-1 transactivation. The data in Fig. 1 *a* shows that antigenic cross-linking of the FcεR1 using KLH-DNP strongly induces Elk-1 transactivation in a dose-dependent manner. In addition, the phorbol ester PdBu that directly activates PKC, induces ninefold induction of Elk-1 transactivation over basal levels. Fig. 1 *a* also shows that elevation of intracellular calcium levels using the ionophore Ionomycin is not sufficient for Elk-1 activation. Stimulation by FcεR1 or the phorbol ester PdBu does not induce LexA OPtk.CAT activity in the absence of a coexpressed LexA Elk-1C fusion protein (Fig. 1 *b*).

FcεR1 ligation is known to activate PKC, and previous studies have established that PKC mediated signals are im-

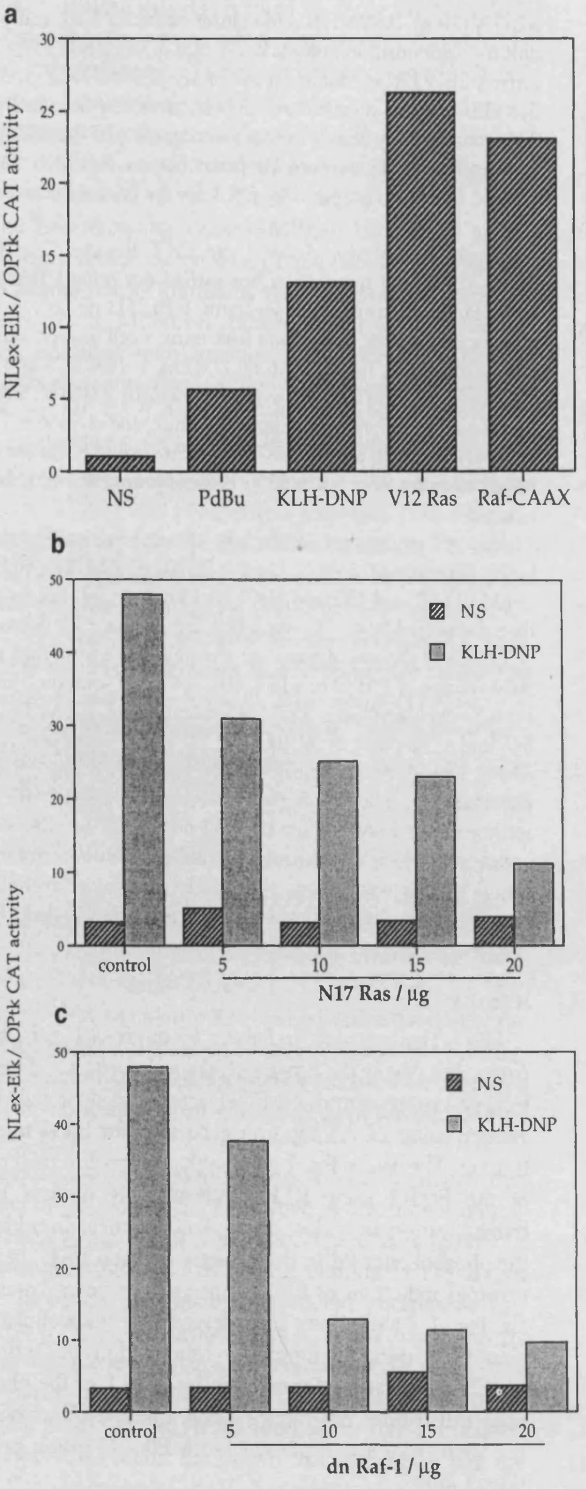

Figure 2. (*a*) Activated Ras or Raf are sufficient for Elk-1 activation in mast cells. 1×10^7 cells per point were transfected with either the Elk-1 reporter system alone or in combination with 10 μg pEF-V12Ras or 10 μg pEF Raf-CAAX. Cells transfected with activated mutants were left unstimulated, while control cells were exposed to either 50 ng/ml PdBu or 1 μg/ml IgE/500ng/ml KLH-DNP in the concentrations indicated above. (*b* and *c*) FcεR1 activation of Elk-1 is dependent on Ras. 1×10^7 cells per point were transfected with either the Elk-1 reporter system alone (*control*), or in combination with 15 μg of either RSV N17Ras or pEF dn Raf-1. Cells were recovered and left unstimulated (*NS*) or exposed to IgE/KLH-DNP as described.

portant for FcεR1 induction of mast cell degranulation. PKC activation is therefore a candidate mechanism for FcεR1 induction of Elk-1. We used a broad-spectrum inhibitor of PKC isozymes, Ro-318425 (30), to assay the contribution of PKC to Elk-1 induction by the FcεR1. Ro-318425 inhibits the activity of the major PKC isozymes shown to translocate to the plasma membrane following FcεR1 ligation. The data in Fig. 1 *c* show that a 500 nM dose of Ro-318425 inhibits PdBu activation of Elk-1 by 85%. In contrast, FcεR1 stimulation can potently induce Elk-1 transactivation, despite the presence of Ro-318425.

The data in Fig. 1 *a* demonstrate that signals derived from the FcεR1 regulate the transcriptional activity of Elk-1. The insensitivity of FcεR1 stimulation of Elk-1 to the PKC inhibitor Ro-318425 suggests that PKC-mediated signals are not essential for Elk-1 regulation by the FcεR1. Raising of intracellular calcium levels by ionomycin treatment or the mimicking of this effect by the cotransfection of an activated mutant of the calcium-dependent phosphatase calcineurin (data not shown) did not induce Elk-1 activity. Hence, we investigated candidate noncalcium/PKC pathways for Elk-1 activation.

Elk-1 Activation in Mast Cells Is Induced by Constitutively Active Mutants of Ras and Raf-1. Elk-1 could be a target for a kinase cascade initiated by Ras. The prototypical kinase cascade transducing Ras signals is the Raf-1/MEK/Erk pathway, and there is a precedent for Elk-1 activation via this mechanism in the fibroblast (31). To explore the role of Ras and Raf-1 signals in Elk-1 activation in mast cells, we examined the effects of activated Ras and Raf-1 mutants on Elk-1 activity in mast cells. The Raf-1 construct used here is a fusion protein of Raf-1 with a CAAX box motif that targets Raf-1 to the plasma membrane, rendering the exogenously expressed Raf-1 constitutively active (32). In these experiments, plasmids bearing the indicated mutants were cotransfected with the LexA Elk-1C fusion protein expression plasmid and the LexA OP.tkCAT reporter gene. The data in Fig. 2 *a* show that activated forms of both Ras and a Ras effector, Raf-1, are capable of inducing Elk-1 transactivation in RBL2H3 cells in the absence of other stimuli.

In additional experiments we investigated whether FcεR1 induction of Elk-1 is dependent on the activation of a Ras cascade using dominant inhibitory mutants. The N17 mutant of Ras acts to sequester guanine nucleotide exchange factors from endogenous pools of the GTPase, and maintains Ras in its GDP-bound, inactive state. The dominant inhibitory (dn) Raf-1 mutant has the COOH-terminal kinase domain truncated; this mutant binds to activated Ras.GTP and prevents its interaction with effector proteins. Figs. 2 *b* and *c* show the effect of dn mutants of Ras and Raf-1 on FcεR1 induction of Elk-1 transactivation. FcεR1 induction of Elk-1 activity is inhibited by expression of either N17Ras or dnRaf-1, in a manner that is dose dependent on the amount of dominant negative plasmid used for cotransfection. At 20 μg cotransfected N17Ras or

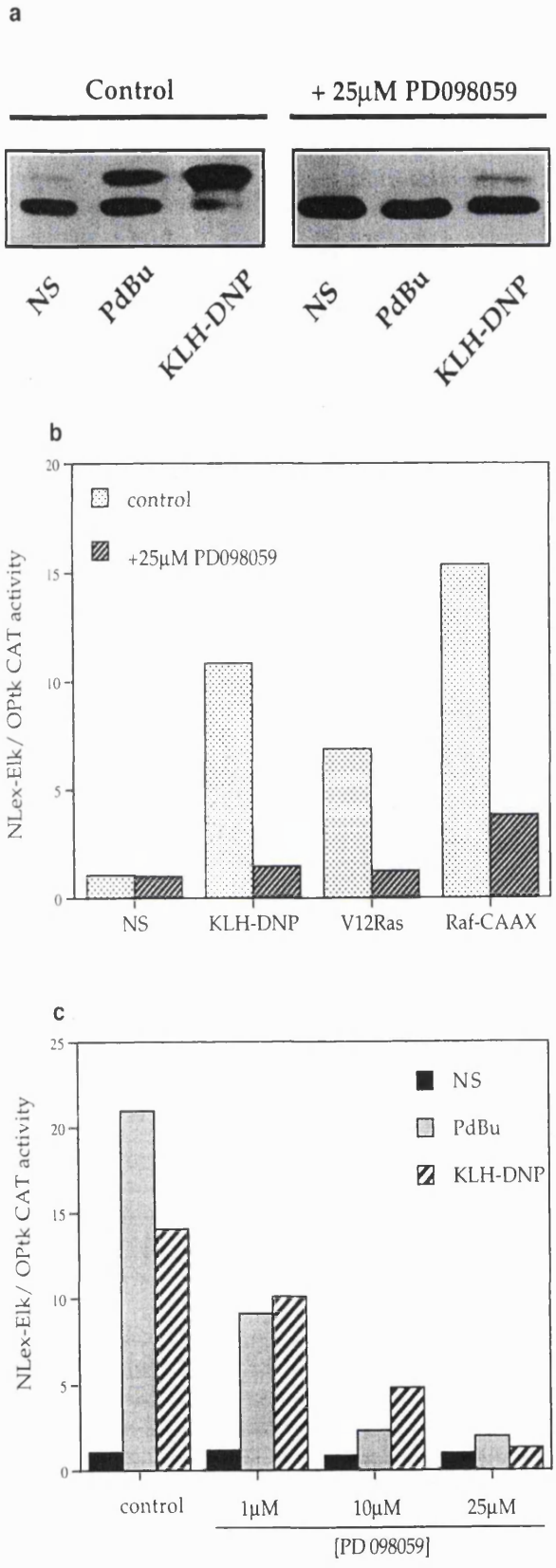

Figure 3. (*a*) PD098059 inhibits MEK activation of Erk in mast cells. 1×10^6 RBL2H3 per point were incubated for 30 min at 37°C with DMSO (*control*) or 25 μM PD098059. Stimulations were for 10 min with either 50 ng/ml PdBu or, after priming with IgE anti DNP, 10 min 500 ng/ml KLH-DNP. Western blotting for Erk was carried out as described in Materials and Methods. (*b*) Induction of Elk-1 via activated Ras and Raf-1 is sensitive to inhibition of MEK. 1×10^7 cells per point were transfected with either the Elk-1 reporter system alone, or in combination with 10 μg pEF V12Ras or 10 μg pEF Raf-CAAX. Control cells were left unstimulated (*NS*) or exposed to IgE/KLH-DNP. Cotransfected cells were left unstimulated. Preincubation with 25 μM PD098059 or DMSO was for 30 min before stimulation. (*c*) Induction of Elk-1 activity by FcεR1 or PdBu is sensitive to inhibition of MEK. 1×10^7 cells per point were transfected with the Elk-1 reporter system and recovered for 6 h. Cells were preincubated for 30 min with either DMSO or the indicated concentrations of PD098059. Cells were left unstimulated (*NS*), exposed to IgE/KLH-DNP, or PdBu as described.

dnRaf-1, the percentage inhibitions of FcεR1 induction of Elk-1 transactivation were 74 and 79%, respectively. The sensitivity of FcεR1 induction of Elk-1 to dominant inhibitory mutants of both Ras and Raf-1 suggests that the "classical" pathway for MAP kinase stimulation is involved in Elk-1 activation in RBL2H3 cells. In this pathway, Ras.GTP induces membrane targeting and activation of Raf-1, that acts subsequently on Erk members of the MAP kinase family via the Erk-activating kinase MEK. However, the dn Raf-1 mutant not only prevents interactions between activated Ras and endogenous Raf-1, but also blocks Ras binding to other effector molecules. Thus, to explore the role of the MEK/Erk2 pathway in Elk-1 regulation more directly, we used PD098059, an inhibitor of the Erk2 stimulatory kinase, the MAP kinase kinase MEK.

The specificity of PD098059 as a MEK inhibitor has been previously described (33). Nevertheless, in initial experiments we verified that PD098059 was an effective MEK inhibitor in RBL2H3 cells. The phosphorylation of Erk by MEK results in decreased electrophoretic mobility of Erk in SDS-PAGE. Fig. 3 *a* shows an Erk mobility shift assay visualized by pan-Erk Western blot. In control cells (*left*), PdBu or FcεR1 stimulation cause the appearance of an hyperphosphorylated Erk population. The application of 25 μM PD098059 (*right*) efficiently inhibits the activation of Erk by MEK in mast cells. Accordingly, we examined the effect of PD098059 on induction of Elk-1 transactivation in mast cells. As shown in Fig. 3 *b*, the activation of Elk-1 induced by cotransfection of Raf-CAAX and V12Ras constructs is inhibited by PD098059. Hence, these mutants are indeed reflecting the use of a Raf-1/Erk pathway to target Elk-1. Fig. 3 *c* shows that the induction of Elk-1 transactivation by both PdBu and FcεR1 cross-linking is inhibited by PD098059 in a dose-dependent manner. These data confirm that the Raf-1/MEK cascade is the critical Ras effector pathway for FcεR1 activation of Elk-1.

NFAT Is a Target for a Ras Effector Pathways Distinct from Raf-1/MEK in the Mast Cell. FcεR1 stimulation results in the activation of cytokine genes, the products of which play important roles both locally in inflammation and systemically. Transcription factors of the NFAT family are important for cytokine gene induction in a variety of cells and a well characterized target for NFAT family proteins in an activated mast cell is the gene for IL-4 (25). FcεR1 triggering has been shown to induce NFAT DNA binding ac-

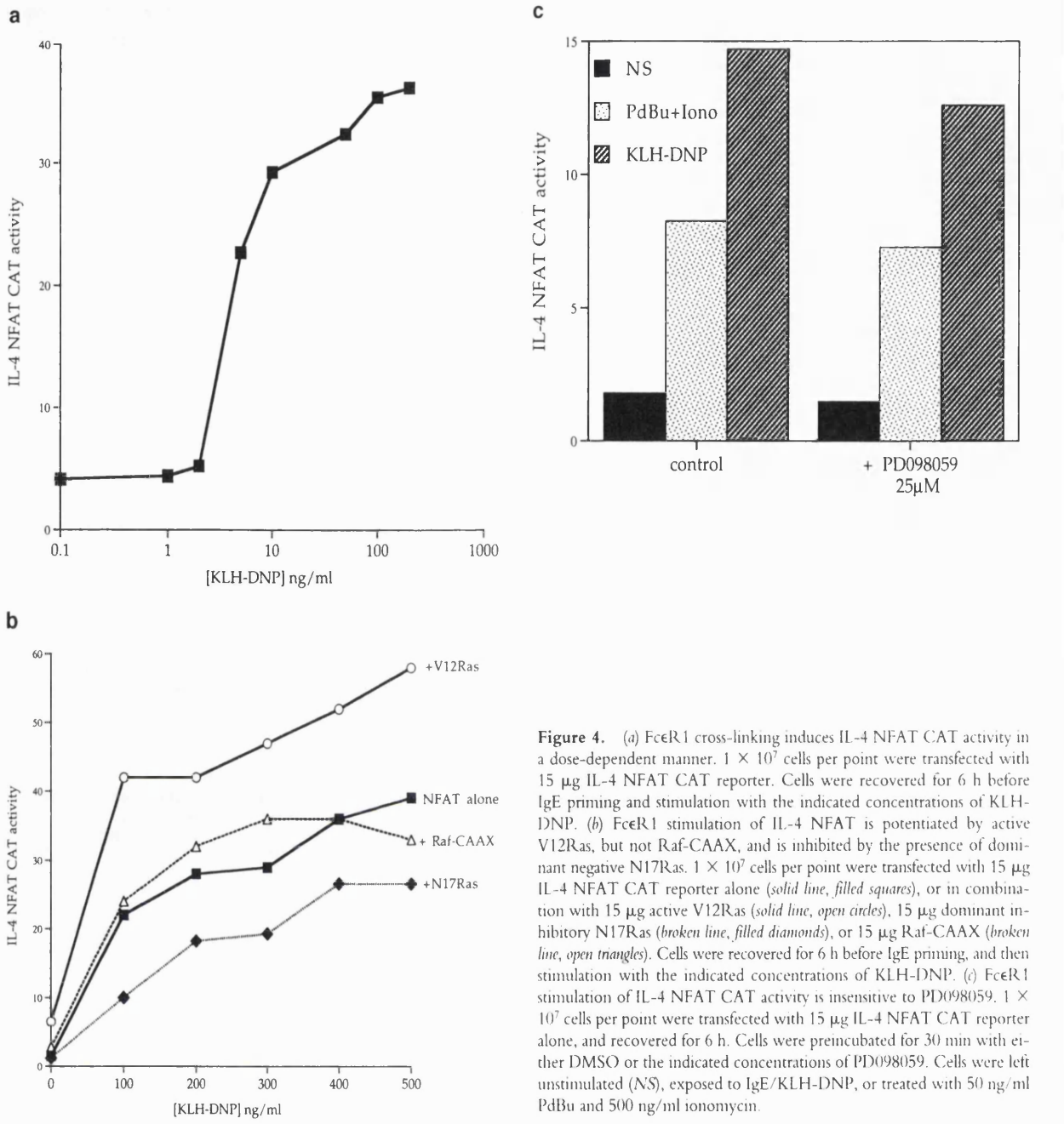

Figure 4. (*a*) FcεR1 cross-linking induces IL-4 NFAT CAT activity in a dose-dependent manner. 1×10^7 cells per point were transfected with 15 μg IL-4 NFAT CAT reporter. Cells were recovered for 6 h before IgE priming and stimulation with the indicated concentrations of KLH-DNP. (*b*) FcεR1 stimulation of IL-4 NFAT is potentiated by active V12Ras, but not Raf-CAAX, and is inhibited by the presence of dominant negative N17Ras. 1×10^7 cells per point were transfected with 15 μg IL-4 NFAT CAT reporter alone (*solid line, filled squares*), or in combination with 15 μg active V12Ras (*solid line, open circles*), 15 μg dominant inhibitory N17Ras (*broken line, filled diamonds*), or 15 μg Raf-CAAX (*broken line, open triangles*). Cells were recovered for 6 h before IgE priming, and then stimulation with the indicated concentrations of KLH-DNP. (*c*) FcεR1 stimulation of IL-4 NFAT CAT activity is insensitive to PD098059. 1×10^7 cells per point were transfected with 15 μg IL-4 NFAT CAT reporter alone, and recovered for 6 h. Cells were preincubated for 30 min with either DMSO or the indicated concentrations of PD098059. Cells were left unstimulated (*NS*), exposed to IgE/KLH-DNP, or treated with 50 ng/ml PdBu and 500 ng/ml ionomycin.

tivity (26), and we investigated FcεR1 mechanisms for NFAT induction in mast cells using a reporter construct comprising a trimerized NFAT site derived from the murine IL-4 promoter.

Fig. 4 *a* shows that FcεR1 cross-linking induces IL-4 NFAT activity in a manner dose dependent on antigen. The PKC inhibitor Ro-318425 did not affect the FcεR1 activation of NFAT (data not shown). To address whether there is a role for Ras in the FcεR1 regulation of NFAT, we cotransfected active and dominant inhibitory Ras mutants with the IL-4 NFAT-CAT reporter gene. Expression of active V12Ras induced a weak increase in the basal activity of the NFAT reporter gene, and robustly potentiated FcεR1 induction of NFAT. Conversely, expression of N17Ras inhibited the NFAT response to FcεR1 (Fig. 4 *b*). Hence, cotransfected activated Ras (V12Ras) causes a potentiation of FcεR1 activation of IL-4 NFAT that potently increases the sensitivity of NFAT responses to antigen. The presence of N17Ras consistently inhibits the antigen dose response for IL-4 NFAT CAT induction to half-maximal levels, and supresses the antigen sensitivity of the mast cells for NFAT activation.

Raf-1 was a key effector for activation of Elk-1 in mast cells and expression of membrane targeted, and hence active, Raf-1 (Raf-CAAX) could mimic the effects of V12Ras and potently stimulate Elk-1 transcriptional activity. However, Fig. 4 *b* shows that expression of Raf-CAAX cannot substitute for V12Ras to induce NFAT transcrip-

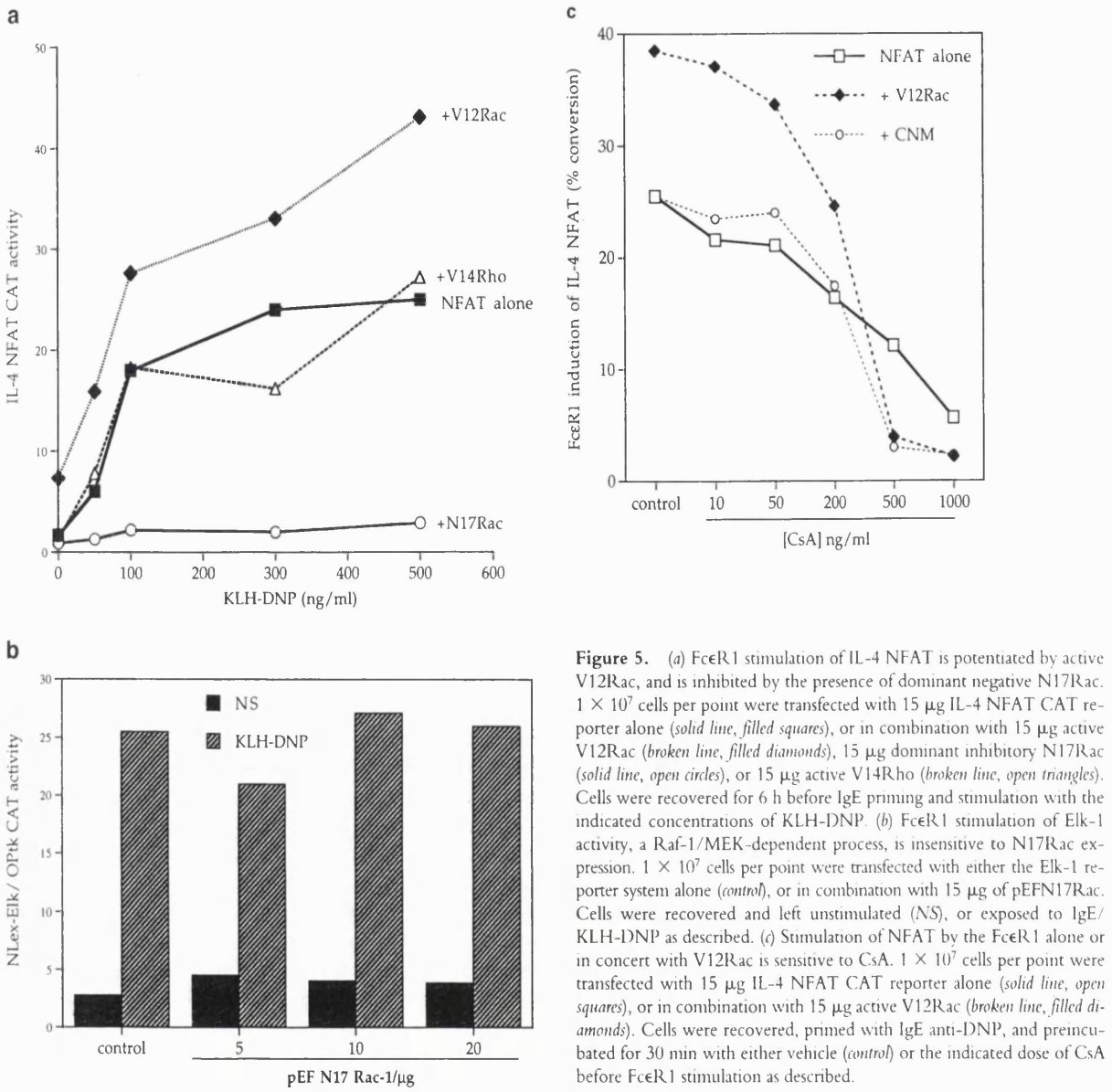

Figure 5. (*a*) FcεR1 stimulation of IL-4 NFAT is potentiated by active V12Rac, and is inhibited by the presence of dominant negative N17Rac. 1×10^7 cells per point were transfected with 15 μg IL-4 NFAT CAT reporter alone (*solid line, filled squares*), or in combination with 15 μg active V12Rac (*broken line, filled diamonds*), 15 μg dominant inhibitory N17Rac (*solid line, open circles*), or 15 μg active V14Rho (*broken line, open triangles*). Cells were recovered for 6 h before IgE priming and stimulation with the indicated concentrations of KLH-DNP. (*b*) FcεR1 stimulation of Elk-1 activity, a Raf-1/MEK-dependent process, is insensitive to N17Rac expression. 1×10^7 cells per point were transfected with either the Elk-1 reporter system alone (*control*), or in combination with 15 μg of pEFN17Rac. Cells were recovered and left unstimulated (*NS*), or exposed to IgE/KLH-DNP as described. (*c*) Stimulation of NFAT by the FcεR1 alone or in concert with V12Rac is sensitive to CsA. 1×10^7 cells per point were transfected with 15 μg IL-4 NFAT CAT reporter alone (*solid line, open squares*), or in combination with 15 μg active V12Rac (*broken line, filled diamonds*). Cells were recovered, primed with IgE anti-DNP, and preincubated for 30 min with either vehicle (*control*) or the indicated dose of CsA before FcεR1 stimulation as described.

tional activity. These data prompted us to investigate the effect of inhibiting the Raf-1/MEK pathway using PD098059 on NFAT transcriptional activity. Fig. 4 *c* shows that the activation of IL-4 NFAT CAT by the FcεR1 is insensitive to the application of PD098059. Hence, in contrast to Elk-1, the critical Ras effector pathway for NFAT regulation in mast cells cannot be Raf-1/Erk.

The Rho Family GTPase Rac-1 Regulates the NFAT Response to FcεR1 Stimulation in Mast Cells. The Rho family GTPase Rac-1 has been shown to play a role in Ras regulation of fibroblast transformation (19) and in T cell antigen receptor regulation of NFAT (34). We therefore investigated the effects of Rho family GTPases in FcεR1 signaling to NFAT. Active mutants of Rac-1 and Rho (V12Rac and V14Rho, respectively) were used to assay whether these GTPases can regulate IL-4 NFAT activation in the mast cell. Fig. 5 *a* shows that expression of the active V12Rac mutant induced an increase in the basal activity of the NFAT reporter gene, and highly potentiated the FcεR1 activation of IL-4 NFAT. To demonstrate the specificity of this effect, we showed that an activated V14Rho had no discernable potentiating effect on IL-4 NFAT induction (Fig. 5 *a*).

The ability of active Rac-1 to potentiate FcεR1/NFAT responses shows that this GTPase has the potential to regulate NFAT activity in mast cells. To examine whether Rac-1 actually plays a role in the NFAT response to FcεR1 stimulation, we cotransfected the NFAT reporter gene with an inhibitory mutant of Rac-1, N17Rac. The data in Fig. 5 *a* show that expression of N17Rac had a marked inhibitory effect on FcεR1 induction of NFAT. As a specificity control, we examined the effect of N17Rac on FcεR1 activation of Elk-1 transcription. Fig. 5 *b* shows that FcεR1 induction of Elk-1 is insensitive to the presence of N17Rac; therefore, we have demonstrated a specific requirement for Rac-1 activity in FcεR1 regulation of NFAT.

Induction of NFAT trancriptional activity in mast cells is dependent on the calcium/calcineurin pathway, and is therefore sensitive to inhibition via the immunosuppressive drug CsA (25). The data in Fig. 5 *c* show that the FcεR1 induction of NFAT is also sensitive to the action of CsA in a dose-dependent manner. Expression of the activated V12Rac mutant, which potentiates FcεR1 induction of NFAT, was unable to reverse the inhibition of the response observed with CsA. These data indicate that FcεR1 induction of NFAT requires at least the convergent action of Rac and CN pathways.

Discussion

The data herein show that the FcεR1 activation of Ras and its effector pathways allow receptor regulation of the transcription factors Elk-1 and NFAT. The diversity of its effector pathways enables Ras to act in a critical position to direct activation of various nuclear targets. We have shown that Ras signals are thus necessary and sufficient for FcεR1 transcriptional activation of Elk-1, a transcription factor important in the context of the serum response element which is a regulatory component of immediate early gene promoters (31). Ras signals also play a role in FcεR1 regulation of NFAT complexes, although they are not sufficent for this response. In this report we have also identified NFAT as a target for Rac-1 signaling pathways in mast cells. Expression of active Rac-1 protein dramatically potentiates FcεR1 induction of NFAT, whereas expression of an inhibitory Rac-1 mutant severely abrogates the NFAT response to FcεR1 stimulation. These data place the GTPases Ras and Rac-1 in a critical position regulating the nuclear component of mast cell end function.

The SRE is responsible for regulation of immediate early genes such as c-fos and egr-1, and has been shown to be a convergence point for varied signaling pathways including the Ras/MEK pathway in fibroblasts (35, 28). Extensive studies have shown that transcriptional activation of Elk-1 is dependent on its COOH-terminal phosphorylation by members of the MAP kinase family such as Erk-1/2, the Jun (stress-activated) kinase JNK, and the p38 kinase (28, 36). Despite the potential for Elk-1 to be targeted by multiple MAP kinases, the data herein show that the Ras effector pathway involved in FcεR1 regulation of Elk-1 absolutely requires the activity of the Erk-activating kinase MEK, acting downstream of Ras and its effector Raf-1. These data show that the Ras/Raf-1/MEK pathway has a dominant role in FcεR1 regulation of Elk-1, reflecting clear parallels between Elk-1 activation following antigen receptor ligation and growth factor stimulation of fibroblasts.

NFAT family members form regulatory complexes which bind cytokine gene promoters (37). Initially described as critical for IL-2 production in T cells, NFAT has also been implicated in transcriptional activation of the genes for IL-4, GM-CSF, and TNFα. NFAT comprises a cytoplasmic component which translocates to the nuclei of stimulated cells and complexes with an inducible nuclear component. The former component is encoded by an extensive gene family including NFATp, NFATc, NFAT-3, and NFAT-4/x. The latter is composed of AP-1 family members, e.g., Fos/Jun. NFATp is a crucial transcription factor for regulation of the IL-4 gene in the T cell (38), and is implicated in transcriptional activation of the IL-4 gene in mast cells (25). IL-4 produced by mast cells after FcεR1 cross-linking is thought to play an important role in the generation of a sustained inflammatory response. Locally, a pulse of mast cell IL-4 stimulates T cells into sustained IL-4 production and subsequent inflammatory cell infiltration. By feeding back on B cells to promote antigen specific IgE production, mast cell–derived IL-4 contributes to the sustained responsiveness of mast cells themselves to antigen/allergen.

Previous studies in T cells have shown that transcriptional activity of the NFAT complex required for IL-2 gene induction is dependent on the concerted action of Ras and calcium/calcineurin signaling pathways (39). Moreover, previous data from our laboratory has shown that multiple Ras effector pathways converge on NFAT in the context of the IL-2 promoter (34). The Raf-1/MEK pathway is one component of TCR induction of NFAT, but there is also a contribution from signals regulated by the GTPase Rac-1 (34). In the context of the activated mast cell, one target for NFAT is the IL-4 gene promoter. We have found that Ras signals are an absolute requirement for FcεR1 regulation of a reporter construct driven by a region of the IL-4 promoter 270 base pairs upstream of the transcriptional start site (Turner, H., and D.A. Cantrell, unpublished observation). The clear implication of this finding is that the IL-4 promoter contains targets for Ras signaling pathways, one of which we establish as NFAT.

Calcium/calcineurin signals are known to be critical for NFAT responses; IL-4 NFAT CAT activity can be strongly induced by an activated mutant of calcineurin or by ionophore treatment (Turner, H., and D.A. Cantrell, unpublished observations). The role of calcium signals in NFAT regulation in the T cell is to induce nuclear translocation of NFAT protein (37, 40, 41). The present data show that in addition to a requirement for calcium/calcineurin, Ras and Rac-1 signaling pathways are important for FcεR1 induction of IL-4 NFAT. While expression of activated Ras could potentiate FcεR1 induction of NFAT, this effect could not be mimicked by stimulation of the Raf-1/MEK pathway using Raf-CAAX. Furthermore, inhibition of MEK/Erk2 activity does not affect FcεR1 activation of NFAT. Hence, we are able to exclude the "classical" Ras/Raf-1/MEK cascade. In comparison with the data in the T cell system, we also observe in mast cells that NFAT integrates multiple signals. In the mast cell, as in the T cell, there is a requirement for both calcium/calcineurin and Rac-1 signals in antigen receptor regulation of NFAT. However, the nature of the Ras signal, which is also required, differs between the two systems. In the mast cell, there is no requirement for Raf-1/MEK activity, while in the T cell, NFAT is regulated by this and another uncharacterized Ras effector pathway (34).

It is recognized that certain cellular responses to Ras re-

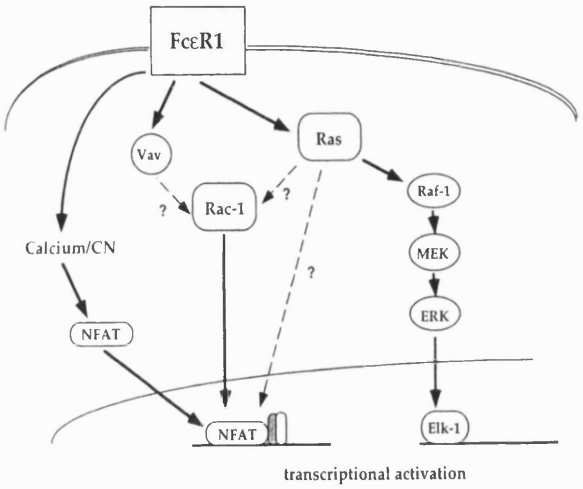

Figure 6. Putative mechanism for FcεR1 action on transcription factor targets in mast cells. Ras signals through the Raf-1/MEK pathway are necessary and sufficient for Elk-1 activation. In contrast, the NFAT complex integrates multiple signals from the FcεR1. Calcium signals induce nuclear translocation of NFAT protein. Ras and Rac-1 GTPases are also implicated in FcεR1 activation of NFAT, acting either in parallel or in series.

quire the activity of other GTPases; notably, Ras mediated transformation of fibroblasts requires signaling pathways mediated by Rac-1 and RhoA (18, 19). The data herein show that IL-4 NFAT activation in mast cells requires the function of Rac-1. The recognition that Rac-1 may play a pivotal role in NFAT responses raises the issue of the identity of Rac-1 effector pathways in mast cells. Proteins that can bind directly to GTP-bound active Rac-1 include members of the p21-associated kinase serine/threonine kinase family (42) and the ribosomal p70S6 kinase (43). Rac-1 has also been shown to activate the MAP kinase family members JNK-1 and p38 (44), making these candidate kinases for transduction of the Rac-1 signal leading to NFAT activation in mast cells.

There is an inhibitor of the p38 kinase available SB203580 (45) which does not modulate NFAT responses to FcεR1 activation (Turner, H., and D.A. Cantrell, unpublished observations). The immunosupressant Rapamycin which inhibits p70S6 kinase also does not inhibit NFAT activation, excluding a role for this kinase in NFAT responses. Hence, it is unlikely that these enzymes are responsible for Rac-1 activation of NFAT. We have observed in preliminary experiments that the FcεR1 activates the MAP kinase JNK-1 (Turner, H., and D.A. Cantrell, unpublished observation, and P. Bauer, personal communication). However, there are no small molecule inhibitors of JNK-1 pathways analogous to the PD098059 MEK inhibitor or the SB203580 p38 inhibitor that can be used to probe the cellular role of JNK-1 in NFAT responses. Therefore, the role of Rac-1 in coupling the FcεR1 to JNK-1 has yet to be examined. Moreover, JNK-1 is by no means the sole remaining candidate for a Rac-1 effector after the elimination of ERK, p38 and p70S6 kinase. There is now a burgeoning body of work on novel Rac-1 effectors including members of the p21-associated kinase family and the cytoskeletal regulatory protein POR-1 (42, 46).

The requirement for both Ras and Rac-1 function for FcεR1 activation of NFAT in mast cells is consistent with a model where Rac-1 is part of a separate FcεR1 signaling pathway that converges with Ras signals on IL-4 NFAT to fully activate the transcription factor complex. Nevertheless, it is equally possible to envisage a mechanism in which Rac-1 acts as a downstream effector for Ras. For example, Vav is a putative Rac-1/RhoA guanine nucleotide exchange protein (21) that is tyrosine phosphorylated in response to FcεR1 ligation (20), and is able to stimulate Rac-1–mediated JNK-1 activation in fibroblasts (22). Vav would thus be a candidate molecule for coupling the FcεR1 to Rac-1 responses. This model is analogous to the links between Ras and Rac-1 that have been documented in fibroblasts where Rac-1 couples Ras to the signaling pathways that control rearrangements of the actin cytoskeleton (47).

It is difficult to resolve these various possibilities (summarized in Fig. 6), and they are not mutually exclusive; Ras and Rac-1 may integrate signals from multiple inputs in the mast cell. This may be true both in terms of multiple intracellular signals generated by antigen receptor ligation, and in terms of reactivity to different extracellular stimuli. Certainly, Ras is not only activated by antigen receptor triggering in mast cells, but can be activated by cytokines such as IL-3 and GM-CSF (48). Activation of Ras-stimulatory pathways by integrins, which are likely to be involved in mast cell adherence to a tissue substratum, has also been documented (49). It is reasonable to expect that during the early course of an inflammatory response, mast cells would be exposed to Ras-activating cytokines such as IL-3 and GM-CSF produced by antigen activated T cells. The effect of Ras activation on IL-4 NFAT activity is essentially that of altering the antigen receptor dose response threshold. Hence, exposure of mast cells to Ras-activating cytokines can be viewed as an important priming step which increases the responsiveness of mast cells to a given antigen dose.

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