Molecular Mechanisms Governing Fcγ Receptor Mediated Signal Transduction

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Dedicated to my Dad

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Abstract

Receptors for the constant region of immunoglobulin G (FcγRs) expressed on myeloid cells play a pivotal role in the clearance of immune complexes and cell activation. In this thesis, the human monoblastic cell line, U937, has been used as a model system to study the coupling of Fcγ receptors to specific signal transduction events. These cells are known to express both the high affinity receptor, FcγRI, and the low affinity activation receptor, FcγRIIa. Previous work has shown that the nature of the signalling cascades initiated by FcγRI fundamentally changes as U937 cells differentiate from a monocyte to a more macrophage phenotype. This thesis describes mechanisms that underlie this developmental switch.

In IFNγ primed U937 cells, FcγRI is coupled to the activation of phospholipase D (PLD). As the subsequent calcium mobilisation is independent of measurable inositol trisphophate production and is not coupled to calcium influx, we examined a potential role for the 5' inositol phosphatase, SHIP, which has been characterised to block both PLCγ activation and calcium influx in mast and B-cells. Here, I demonstrate that following immune complex stimulation, SHIP is tyrosine phosphorylated and associates with the adapter protein, Shc, and immune complexes at the plasma membrane. In B-cells and mast cells, SHIP is recruited by the inhibitory Fc receptor, FcγRIIb. However, in IFNγ primed U937 cells, no FcγRIIb could be found. Interestingly, FcγRIIb expression was induced following differentiation of U937 cells to a more macrophage phenotype using dibutyryl cyclic AMP (dbcAMP). This was surprising as here immune complexes activate PLCγ and induce calcium influx. In these cells, FcγRIIb appears to modulate rather than inhibit immune complex activation.

In IFNγ primed U937 cells, activation of PLCγ was coupled to FcγRIIa but not to FcγRI. The mechanism of this coupling was investigated. I demonstrate that FcγRIIa recruits preformed SLP76:PLCγ complexes to the plasma membrane whereas FcγRI does not. I further show that FcγRI and FcγRIIa induce distinct activation patterns of the MAP kinase cascades.

Finally I have demonstrated that the signalling pathway initiated by FcγRI depends entirely on the recruited accessory molecule. Thus, in IFNγ primed cells, FcγRI physically and functionally associates with the γ-chain. Conversely, in dbcAMP differentiated cells, FcγRI no longer uses the γ-chain but is coupled to PLCγ activation by the recruitment of FcγRIIa. The γ-chain in these differentiated cells is preferentially associated with the IgA receptor and appears to define receptor coupling to PLD.

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Abbreviations

ADCC	Antibody dependent cell mediated cytotoxicity
ARF	Adenosine 5'diphosphate ribosylating factor
ATP	Adenosine 5'-triphosphate
BCR	B cell receptor
BLNK	B-cell LiNKer protein
BSA	Bovine serum albumin
cDNA	Complementary DNA
CRP	C-reactive protein
CTLA	Cytotoxic T-lymphocyte antigen
DAG	Diacylglycerol
dATP	Deoxy Adenosine 5'-triphosphate
dbcAMP	Dibutyryl cyclic adenosine 5'-triphosphate
ddH_2O	Double distilled water
dGTP	Deoxy Guanosine 5'-triphosphate
DMEM	Dulbecco's modified eagles medium
DNA	Deoxy-ribonucleic acid
dTTP	Deoxy thymidine 5' triphosphate
ECL	Enhanced chemiluminesence
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
F(ab)	Antigen binding fragment of an immunoglobulin
Fc	Constant region of an immunoglobulin
FcαR	Receptor for the constant region of IgA
FcεR	Receptor for the constant region of IgE
FcγR	Receptor for the constant region of IgG
FcμR	Receptor for the constant region of IgM
FcRn	Neonatal Fc receptor
FCS	Foetal calf serum
FITC	Fluoroscein isothiocyanate

GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanine 5'-diphosphate
GEF	guanine exchange factor
GPI	Glycosyl phosphatidyl inositol
GSK-3	Glycogen synthase kinase
HRP	Horse radish peroxidase
IFN-γ	Interferon-gamma
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgSF	Immunoglobulin super family
IKK	IκB kinase
IL	Interleukin
$InsP_3$	Inositol trisphosphate
$InsP_4$	Inositol tetrakisphosphate
IPTG	Isopropylthiogalactoside
ITAM	Immunoreceptor tyrosine based activation motif
ITIM	Immunoreceptor tyrosine based inhibitory motif
JNK/SAPK	c-Jun N terminal kinase
KIR	Killer inhibitory receptor
KIR	Killer inhibitory receptors
LAT	Linker for activation of T-cells
LIR	Leukocyte inhibitory receptor
LY	LY294002
mAb	Monoclonal Antibody
MAPK	Mitogen activated protein kinase
MEK	MAPK/ERK kinase
MEKK	MAPK/ERK kinase kinase
MHC	Major histocompatability complex

MKK	MAP kinase kinase
MKKK	MAP kinase kinase kinase
MLB	Magnesium lysis buffer
mRNA	messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear Factor κB
NK cell	Natural Killer cell
PA	Phosphatidic acid
PAK	p21 activated kinase
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PC-PLD	Phosphatidylcholine-phospholipase D
PCR	Polymerase chain reaction
PDK	PIP_3 dependent kinase
PH domain	Pleckstrin homology domain
PI 3-kinase	Phosphatidyl inositol 3 kinase
PI	Phosphatidyl inositol
PIP	Phosphatidyl inositol phosphate
PIP_2	Phosphatidylinositol bisphosphate
PIP_3	Phosphatidyl inositol trisphosphate
PKB	Protein kinase B
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PTB domain	Phosphotyrosine binding domain
RBD	Ras binding domain
RBL	Rat basophilic leukaemia
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase- polymerase chain reaction
SCaMPER	Sphingolipid Ca^{++} release mediating protein of ER

SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylmide gel electrophoresis
SH2 domain	Src homology 2 domain
SH3 domain	Src homology 3 domain
SHIP	SH2 containing inositol phosphatase
SHP	SH2 containing protein tyrosine phosphatase
sIg	Surface Immunoglobulin
SLP-76	SH2 domain containing linker protein - 76 kDa
T4-PNK	T4-polynucleotide kinase
TBS	Tris buffered saline
TBST	Tris buffered saline containing Tween-20
TCR	T cell receptor
TEMED	N,N,N',N',-Tetramethylethylenediamine
TGF-β	Transforming growth factor-beta
TNF-α	Tumour necrosis factor-alpha
WM	Wotmannin
XLA	X-linked agammaglobulinaemia

Chapter 1

General Introduction

1.1 Fc Receptors

1.1.1. Fc receptors and the immune response

Immune responses are initiated when an individual encounters antigenic material, generally in the form of an infectious agent. A major facet of this immune response is the production and secretion of antigen specific antibodies (immunoglobulins) by B-lymphocyte derived antibody secreting cells. Secreted immunoglobulins (Ig) serve to bind the antigenic material to form immune complexes. These immune complexes are then targeted to cells bearing specific receptors which recognise Ig. These receptors are collectively known as Fc receptors as they bind to the constant region (Fc) of Ig, while the variable region (Fab) binds to the antigen (Figure 1.1). Fc receptors thus provide a bridge between the humoral (extracellular) and cellular arms of the immune response.

1.1.2. Fc Receptors - overview

Fc receptors (FcRs) recognise the constant region (Fc) of immunoglobulins (Ig). There are five main classes of Ig; IgG, IgA, IgE, IgM and IgD. Each class of Ig has a different Fc region and these are recognised by a specific set of receptors. Thus, IgG is recognised by FcγRs, IgA by FcαRs, IgE by FcεRs and IgM by FcμRs (Raghavan & Bjorkman, 1996). Several different classes of structurally and functionally distinct Fc receptors have been identified. These include the lectin like IgE receptors (CD23) and the immunoglobulin transporters, exemplified by the neonatal IgG transporter (FcRn) and the polyimmunoglobulin transporter for IgM and IgA (Raghavan & Bjorkman, 1996). The largest and best characterised group of Fc receptors, however, belong to the Ig domain super family (IgSF) of receptors (Ravetch & Kinet, 1991, Vandewinkel & Anderson, 1991, Daeron, 1997b, Daeron, 1997a). This group includes the high affinity IgE receptor

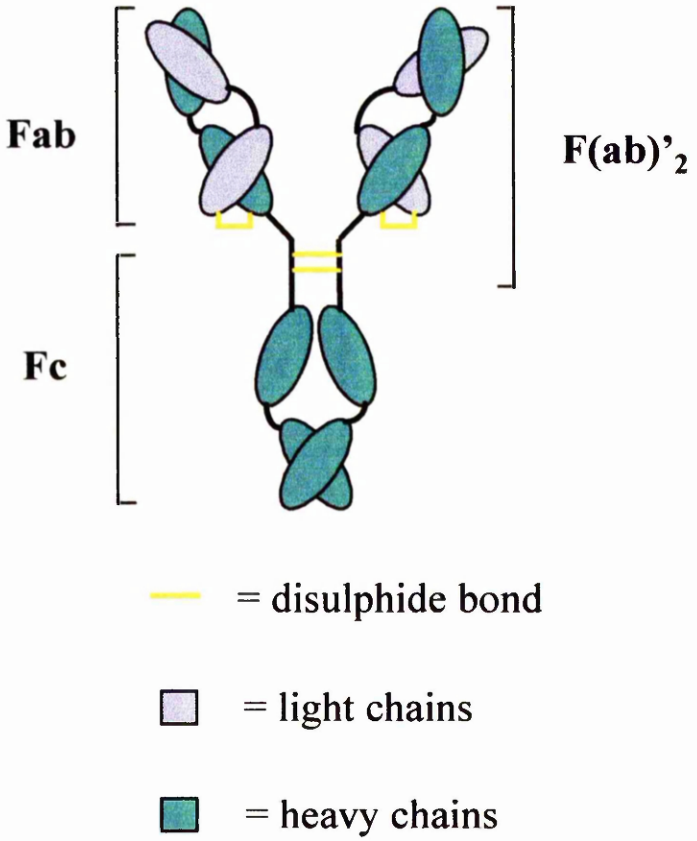


Figure 1.1. Schematic Structure of an Ig molecule. Each coloured oval symbolises a complete IgSF domain. The two heavy chains (green) each comprise four IgSF domains. The two light chains each comprise two IgSF domains. The four chains are linked by disulphide bonds as indicated.

(FcεRI), the high affinity receptor for IgA (FcαRI) and high and low affinity receptors for IgG (FcγR). These receptors generally exist as multisubunit receptor complexes, comprising a ligand binding α-chain and associated signalling chains (i.e. γ-chain). The ligand binding chains of these receptors, with the exception of FcγRIIIb (section 1.1.3.3) are all type I membrane glycoproteins comprising highly conserved extracellular immunoglobulin domains of the V class, and less conserved transmembrane and cytoplasmic domains. The main focus of this thesis lies on Fcγ receptors of the IgSF family (Table 1.1). However, due to analagous functions and mechanisms of action, FcαRI and FcεRI will also be discussed in some detail (Table 1.1).

1.1.3. Fcγ receptors

Structurally and functionally, the Fcγ receptors represent the most diverse group of Fc receptors. At least 8 separate genes have been identified in man, all mapped to chromosome 1, with each gene giving rise to multiple splice variants (Figure 1.2). Fcγ receptors can be divided into three main subgroups, based on their affinity for IgG and mAb reactivity, termed FcγRI, FcγRII and FcγRIII (Table 1.1) (Hulett & Hogarth, 1994, Ravetch, 1994).

1.1.3.1. FcγRI (CD64)

FγRI is a high affinity receptor which binds monomeric IgG at physiological concentrations (Allen & Seed, 1989). FcγRI is constitutively expressed on monocytes and macrophages where its expression is tightly regulated by IFN-γ (Harris *et al.*, 1985). FcγRI expression can also be induced by IFN-γ on neutrophils and eosinophils (Hulett & Hogarth, 1994, van de Winkel & Capel, 1993). The unique ability of FcγRI to bind monomeric IgG may reflect a role for this receptor in triggering effector functions at low concentrations of IgG (Shen, Guyre & Fanger, 1987). It has been proposed that the high affinity binding of FcγRI is conferred by the presence of the third extracellular domain (Allen & Seed, 1989) (Figure 1.2). Three human FcγRI genes have been identified and mapped to chromosome 1q21.1 (Hulett & Hogarth, 1994, van de Winkel & Capel, 1993). Only one of these genes, FcγRIa, encodes a membrane anchored high affinity receptor.

This receptor comprises three extracellular IgSF domains, (unlike all other FcRs which have two IgSF domains) a short transmembrane region and a short cytoplasmic domain. Stop codons in the coding region of the third IgSF domain (membrane proximal) of the other two genes, FcγRIb and FcγRIc, predict soluble forms of FcγRI. Alternative splicing of FcγRIb has also been proposed to generate a truncated low affinity membrane bound receptor lacking the third extracellular domain (Ernst *et al.*, 1992) (Figure 1.2).

The cytoplasmic tail of FcγRI contains no known signalling motif (Allen & Seed, 1989). To couple to signalling pathways, FcγRI associates at the cell surface with γ-chain homodimers (Ernst, Duchemin & Anderson, 1993, Scholl & Geha, 1993, Ra *et al.*, 1989). The γ-chain, initially found in association with FcεRI, contains a cytoplasmic immunoreceptor tyrosine based activation motif (ITAM) responsible for the recruitment of tyrosine kinase dependent signalling pathways (discussed in Section 1.2.1.1) (Cambier, 1995).

1.1.3.2. FcγRII (CD32)

The FcγRII receptors represents by far the most widely distributed and functionally diverse group of human IgG receptors (Stuart *et al.*, 1989, Brooks *et al.*, 1989). Three human FcγRII genes have been identified, FcγRIIa, FcγRIIb and FcγRIIc; each giving rise to multiple splice variants. All three genes are mapped to chromosome 1q23-24. All isoforms of FcγRII, with the exception of FcγRIIa2 (which encodes a soluble form of FcγRIIa), are integral type I membrane proteins comprising two extracellular IgSF domains, a short membrane spanning region and a cytoplasmic region of variable length (44-77 amino acids) (Figure 1.2). Unlike FcγRI, FcγRII is a low affinity receptor, incapable of binding monomeric IgG. Physiologically, this low affinity ensures that FcγRII is only engaged by IgG that has been aggregated by multivalent antigens (Raghavan & Bjorkman, 1996, van de Winkel & Capel, 1993, Hulett & Hogarth, 1994).

While the extracellular domains of all FcγRII isoforms are highly homologous (>95%), the cytoplasmic tails are highly divergent. The divergence of the FcγRII proteins exemplifies how near identical receptors can give rise to highly divergent biological responses (Brooks

et al., 1989). Unlike FcγRI and FcγRIII, the FcγRII receptors encode signalling motifs within their cytoplasmic tails, and are capable of triggering signalling cascades (Van den Herik-Oudijk *et al.*, 1995a). Differences in the cytoplasmic motifs encoded by the three FcγRII genes specifies recruitment of distinct pathways (Figure 1.2). Thus, FcγRIIa has an ITAM (immunoreceptor tyrosine based activation motif) within its cytoplasmic tail. This motif is capable of triggering tyrosine kinase dependent signalling cascades (discussed in section 1.2.1.2). Conversely, FcγRIIb1 has an ITIM (immunoreceptor tyrosine based inhibitory motif) within its cytoplasmic tail which recruits an inhibitory signalling pathway (discussed in section 1.2.8) (Muta *et al.*, 1994b, Daeron, 1996, Van den Herik-Oudijk *et al.*, 1995a).

Further diversity of function is generated by alternative splicing of the FcγRIIa and FcγRIIb transcripts (Brooks *et al.*, 1989). Thus FcγRIIa gives rise to both a membrane associated receptor (FcγRIIa1 - referred to as FcγRIIa) and a soluble form (FcγRIIa2). FcγRIIb gives rise to three alternatively spliced transcripts (FcγRIIb1-3) (Brooks *et al.*, 1989). FcγRIIb1 and FcγRIIb3 give rise to identical mature proteins though their signal sequences differ. FcγRIIb2 however lacks a cytoskeletal attachment domain responsible for modulating receptor internalisation (Daeron, 1997a) (Figure 1.2). FcγRIIa expression is generally limited to myeloid cells, including monocytes, macrophages, and neutrophils. FcγRIIb isoforms are widely expressed on cells of hematopoietic origin, including B-cells and mast cells, where the inhibitory properties of these receptors have been extensively studied (section 1.2.8).

Interestingly, analysis of the gene structure for FcγRIIc indicates high homology to FcγRIIa at the 3' end and high homology to FcγRIIb at the 5' end implying that this gene resulted from an unequal crossover event between the genes for FcγRIIa and FcγRIIb (van de Winkel & Capel, 1993). The result is a gene product with extracellular domains like FcγRIIb bearing an ITAM containing cytoplasmic tail homologous to FcγRIIa. FcγRIIc expression has recently been reported in natural killer (NK) cells where they are co-expressed with FcγRIIIa (Metes *et al.*, 1994, Metes *et al.*, 1999). Four known splice variants of FcγRIIc have been identified (FcγRIIc1-4) though the biological importance of these variants is unknown.

Table 1.1 Fc receptor ligands, affinities and cell distribution

Receptor	Ligand	Affinity (K_A)	Cell Distribution
FcγRIa CD64	IgG	high 2-5x10^9 M^{-1} IgG1=IgG3>IgG4 IgG2 does not bind	Monocytes, macrophages IFN-γ induced neutrophils, eosinophils
FcγRIIa CD32	IgG	low IgG1=IgG3>IgG2* IgG4 does not bind	monocytes, macrophages, neutrophils, platelets
FcγRIIb CD32	IgG	low IgG1=IgG3>IgG4 * IgG2 does not bind	widely expressed - B-cells, mast cells
FcγRIIc CD32	IgG	low	NK cells
FcγRIIIa CD16	IgG	low	NK cells, macrophages, mast cells
FcγRIIIb CD16	IgG	low	neutrophils
FcαRI CD89	IgA	med/high ~5x10^7 M^{-1}	monocytes, macrophages, neutrophils and eosinophils
FcεRI	IgE	high ~1x$10^{10}$$M^{-1}$	mast cells, basophils

* For low affinity receptors, binding refers to immune complexes, not monomeric Ig.

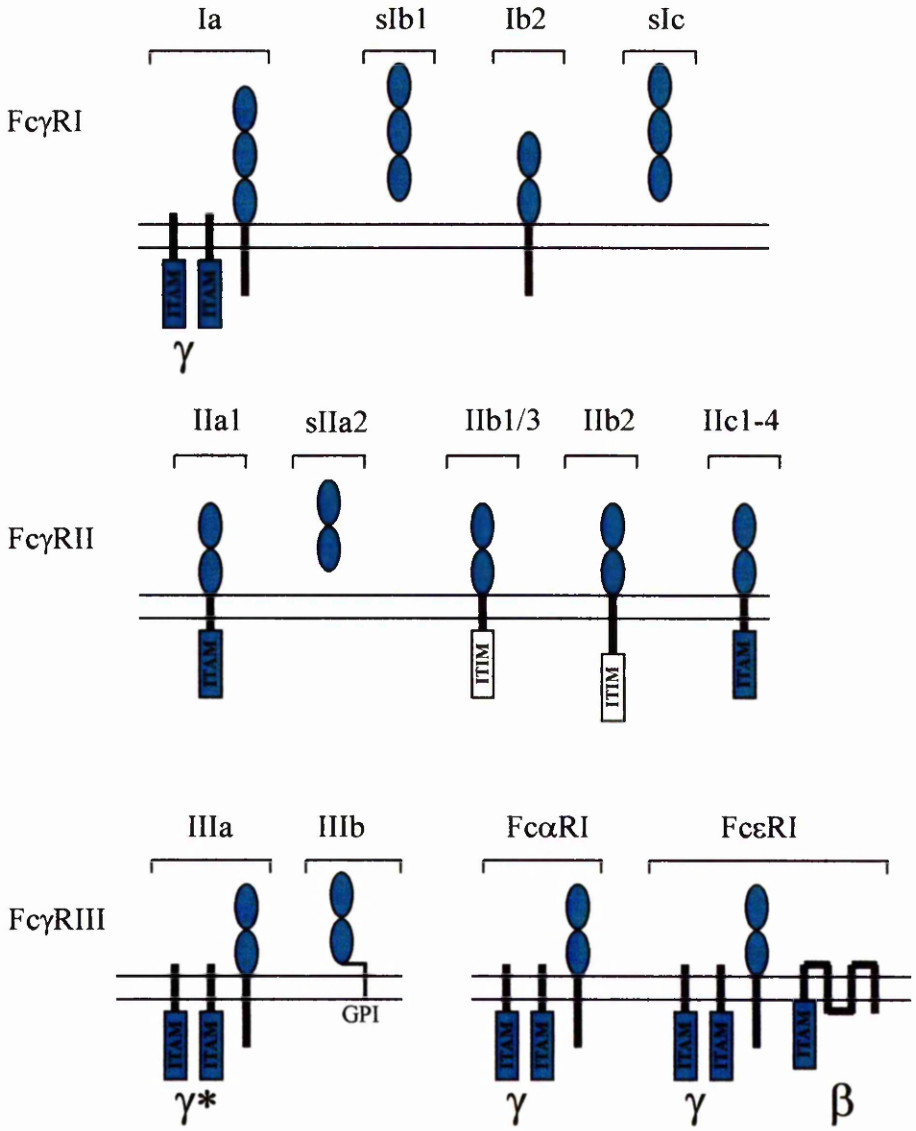


Figure 1.2. Fc receptors and their associated signalling chains. ITAM and ITIM motifs are indicated. γ-chain associates with FcγRI, FcγRIII, FcαRI and FcεRI as indicated (γ). * indicates that FcγRIII also associates with ζ-chain. The β-chain of FcεRI is indicated β.

Importantly, differences between mouse and human Fcγ receptor expression are most notable for FcγRII. In mouse only one gene for FcγRII exists, encoding the inhibitory FcγRIIb receptor. As in humans this gene gives rise to alternative splice variants. No ITAM bearing FcγRII receptor isoforms exists in mouse.

1.1.3.3. FcγRIII (CD16)

FcγRIII, like FcγRII, is a low affinity receptor recognising only IgG aggregated to multivalent antigens (Daeron, 1997a, van de Winkel & Capel, 1993, Hulett & Hogarth, 1994). Two, highly homologous genes for FcγRIII exist in man, FcγRIIIa and FcγRIIIb, mapped to chromosome 1q23-24. While both receptors share similar extracellular domains, comprising two IgSF domains, they differ dramatically in their anchoring to the cell surface (Ravetch & Perussia, 1989) (Figure 1.2). Thus, FcγRIIIa is anchored via a single transmembrane region with a short (25 aa) cytoplasmic tail, whereas FcγRIIIb is attached to the membrane via a glycophosphatidyl inositol (GPI) anchor. The receptors also differ in their cellular distribution, with FcγRIIIa expression found on macrophages, natural killer cells (NK) and mast cells while FcγIIIb expression is limited to neutrophils. As with FcγRI, FcγRIIIa associates with γ-chain homodimers though interestingly, FcγRIIIa can also associates with the related ζ-chain in NK cells (Lanier, Yu & Phillips, 1989). The lack of a transmembrane region prevents FcγRIIIb from associating with either γ or ζ chains (Figure 1.2). Of note, mice express only a single form of FcγRIII which is analagous to human FcγRIIIa being anchored by a transmembrane region and associating with the γ-chain (Figure 1.2).

1.1.3.4. Fcγ receptors have different affinities for IgG subtypes

The specific effector mechanisms elicited by immune complexes depends on the Ig class produced. Fcγ receptors bind to the constant region of IgG. However, four subclasses of IgG are produced by both man (IgG1, IgG2, IgG3 and IgG4) and mouse (IgG1, IgG2a, IgG2b and IgG3). In addition to differences in factors such as size and half life, these IgG subtypes have different affinities for specific Fcγ receptors (Table 1.1)] (van de Winkel & Capel, 1993). Thus, FcγRI binds IgG3 and IgG1 with high affinity, IgG4 more weakly

and fails to bind IgG2 at all. Similarly, FcγRII binds both IgG1 and IgG3 complexes. FcγRII is the only Fcγ receptor to recognise IgG2 and this binding is complicated by allelic differences (van de Winkel & Capel, 1993). Interestingly, though the extracellular domains of FcγRIIa and FcγRIIb are 96% identical, these receptors show differences in affinity for IgG4. Thus, FcγRIIb binds IgG4 complexes in addition to IgG1 and IgG3 complexes, whereas FcγRIIa does not.

Thus, cellular response to IgG immune complexes not only depends on the repertoire of Fcγ receptors expressed, but also on the IgG subtype(s) involved. While different antigenic stimuli give rise to the production of different IgG subtypes, the functional outcome of these differences remains unclear.

1.1.4. Fcα receptors

FcαRI (CD89), with the exception of the polyimmunoglobulin transporter, is the only known receptor for IgA(Daeron, 1997a). The gene for FcαRI exists as a single copy, though multiple splice variants have been reported (de Wit *et al.*, 1995). Further, FcαRI is subject to heterogenous glycosylation with the apparent molecular weight varying considerably (45-100 kDa). FcαRI is an integral type I membrane protein with two extracellular IgSF domains, a short transmembrane region and a short cytoplasmic tail (Daeron, 1997a). Expression of this receptor is limited to myeloid cells, with constitutive expression found on monocytes, macrophages, neutrophils and eosinophils. Further, FcαRI expression has also been reported in mesangial cells provoking much interest in a potential role for this receptor in the human kidney disease, IgA nephropathy (Barratt *et al.*, 2000). The receptor possesses medium to high affinity (K_A~$1x10_7$ M^{-1}) and it can thus bind both monomeric and polymeric forms of both IgA1 and IgA2 (Monteiro, Kubagawa & Cooper, 1990). In common with many other Fc receptors, FcαRI has been shown to associate with γ-chain homodimers (Morton *et al.*, 1995) (Figure 1.2). Of interest, no equivalent to human FcαRI has been identified in the mouse.

1.1.5. Fcε receptors

IgE is recognised by both a high affinity receptor (FcεRI) and a low affinity receptor (CD23/FcεRII). These receptors are unrelated. FcεRI belongs to the IgSF family, along with the FcγRs and FcαRI. In contrast, CD23 is a lectin like molecule (Conrad, 1990). The discussion here is limited to FcεRI. The single gene for the FcεRI α-chain is found, clustered with the gene for the γ-chain, on chromosome 1q22. Expression of FcεRI is predominantly limited to mast cells and basophils (Raghavan & Bjorkman, 1996). Due to its central role in mediating allergic responses, this receptor is probably the most studied of the Fc receptors (Metzger, 1999). FcεRI shares significant homology with FcγRs (~40%) and in common with FcγRI, FcγRIII and FcαRI, this receptor associates with the γ-chain (Blank *et al.*, 1989) (Figure 1.2). In addition, FcεRI associates in mast cells with a second signalling chain, the β-chain, which also bears an ITAM motif (section 1.2.1.1) (Blank *et al.*, 1989, Cambier, 1995, Turner & Kinet, 1999).

Interestingly, the mouse low affinity IgG receptor, FcγRIIb, has also been reported to bind IgE with low affinity (Takizawa, Adamczewski & Kinet, 1992). The role of FcγRIIb in the modulation of FcεRI signalling has been the focus of considerable work and will be discussed separately in section 1.2.8.5.

1.1.6. The γ-chain associates with multiple signalling receptors

Aggregation of Fc receptors results in the activation of tyrosine kinases as evidenced by tyrosine phosphorylation of proteins and tyrosine kinase dependent calcium transients. The majority of Fc receptors do not have a cytoplasmic ITAM motif, known to be involved in recruitment of Src kinases. To couple to tyrosine kinases, these receptors must therefore recruit an accessory signalling molecule, and for many of these receptors, the γ-chain fulfills this role (Figure 1.2). The ITAM containing γ-chain, a 7 kDa protein, was originally found in association with FcεRI (Blank *et al.*, 1989). The γ-chain is now known to associate with other Fc receptors including FcγRI (Ernst *et al.*, 1993, Scholl & Geha, 1993), FcγRIII (Lanier *et al.*, 1989) and FcαRI (Morton *et al.*, 1995) as well as the TCR and most recently the collagen receptor glycoprotein VI (Nieswandt *et al.*, 2000). The γ-chain, belongs to the same family of signal transducing molecules as the ζ-chain and

η–chain of the T-cell receptor. These proteins form disulphide linked homo- or heterodimers. The interactions governing receptor:γ-chain interactions differ for each receptor and are discussed further in Chapter 6. Interestingly, some receptors, including FcεRI and FcγRIII require γ-chain association for surface expression (Lanier *et al.*, 1989).

1.1.7. Fc receptor crystal structures

The last year has seen the solution of a number of Fc receptor crystal structures, both alone and in complex with immunoglobulin Fc fragments. These structures have provided valuable insights into the molecular basis of ligand specificity, binding affinity and receptor function. Three independent groups have separately solved the structures of the extracellular domains of FcεRI (Garman, Kinet & Jardetzky, 1999), FcγRIIa (Maxwell *et al.*, 1999) and FcγRIIb (Sondermann, Huber & Jacob, 1999). The overall fold of these receptors is very similar with the two immunoglobulin domains folded at an acute angle to each other, bringing them into contact over a broad area. Interestingly, FcγRIIa crystallized as a dimer and an analysis of the dimerisation interface has suggested this may be a functional interaction. In this model, FcγRIIa dimers bind to a single IgG molecule. FcγRIIa, unlike all other IgSF Fc receptors, bears an ITAM signalling motif within its cytoplasmic tail. While no crystallographic evidence was reported for FcγRIIb dimerisation, a stoichiometry of 2:1 for the receptor:IgG complex has been proposed on the basis of computer modeling and gel filtration data. Solution of FcεRI in complex with the Fc fragment of IgE (Garman *et al.*, 2000) and FcγRIII in complex with the Fc fragment of IgG1 (Sondermann *et al.*, 2000) has indicated a 1:1 ratio of receptor to ligand. Comparison of the two complexes reveals a high degree of structural similarity between the receptor-Fc interactions. It has been proposed that dimerisation of FcγRII, but not FcεRI and FcγRIII, might be a functional requirement, as FcγRIIa and FcγRIIb, unlike all other IgSF Fc receptors, bear signalling motifs in their cytoplasmic domains. As FcεRI and FcγRIII (along with FcγRI and FcαRI) associate with preformed γ-chain dimers, such receptor pairing may not be required for these receptors. The possible heterodimerisation of FcγRIIa with FcγRIIb has also been proposed.

A structure for FcγRI, which contains a third extracellular IgSF domain, has yet to be solved. The high degree of homology between FcγRII and the first two Ig-like domains of FcγRI however suggests that structural similarity is likely. This is supported experimentally as mutant FcγRI, lacking the third extracellular domain, binds IgG with low affinity (Hulett & Hogarth, 1994).

1.1.8. Fc receptor effector functions.

The biological responses triggered by Fc receptor stimulation appears to depend more on the cell type than the receptor. Thus, different receptors will often stimulate the same responses when expressed in the same cell. Conversely, when expressed in different types of cells, Fc receptors tend to trigger a cell type specific response (Daeron, 1997a). The biological responses to immune complexes can be broadly divided into the two categories of internalisation and activation. Internalisation can occur by the process of endocytosis or phagocytosis. Responses resulting from Fc receptor mediated activation include antibody-directed cellular cytotoxicity (ADCC), degranulation, the respiratory burst and induction of cytokine gene transcription.

1.1.8.1. Phagocytosis

Phagocytosis is the uptake of large particles (0.5 μm or greater) which is mediated by reorganisation of the actin-based cytoskeleton and by definition is inhibited by cytochalasins (Silverstein *et al.*, 1989). In higher organisms, phagocytosis is limited to specific phagocytic cells which include monocytes, macrophages and neutrophils. Phagocytosis is initiated by interaction between specialised cell surface receptors and ligand coated particles. Thus, Fc receptor mediated phagocytosis involves the uptake, and subsequent disposal, of immunoglobulin opsonised particles. Following initiation by receptor ligand interactions, local actin rearrangements result in pseudopod extension and particle engulfment. The resulting internalised phagosomes are able to fuse with endosomes and/or lysozomes where the contents are exposed to low pH and hydrolytic enzymes. Antigenic peptide products resulting from degradation may then be presented to T-cells via MHC class II molecules.

FcγRI, FcαRI, FcεRI and FcγRIII are all capable of mediating phagocytosis in a mechanism dependent on association with the ITAM bearing γ-chain. Conversely, FcγRIIa and FcγRIIc are capable of mediating phagocytosis independent of the γ-chain by virtue of the cytoplasmic ITAMs (Figure 1.2) (Ravetch, 1994, Daeron, 1997a).

1.1.8.2. Endocytosis

Endocytosis is the process of internalising small particles or soluble complexes through receptor ligand interactions. Generally this process occurs through formation of clathrin coated pits at the plasma membrane containing ligand-receptor complexes. These pits bud off into the cytoplasm where the internalised vesicles fuse with early endosomes. Unlike phagocytosis, endocytosis does not require actin polymerisation and so is unaffected by cytochalasins. Most Fc receptors are capable of mediating the endocytosis of immune complexes. Following endocytosis, immune complexes are targeted to appropriate subcellular compartments for antigen presentation and/or immune complex degradation. Interestingly, the requirements for Fc receptor mediated endocytosis differ depending on the specific Fc receptor involved. Thus, FcγRI and FcγRIIa internalise immune complexes independent of accessory molecules whereas murine FcγRIIIa requires association with the γ-chain (Harrison *et al.*, 1994, Anderson *et al.*, 1990, Amigorena *et al.*, 1992b). Further, FcγRIIb1, inhibits immune complex endocytosis while its splice variant, FcγRIIb2 (which lacks a short cytoplasmic signal sequence) mediates efficient endocytosis (Daeron, 1997a). The factors which govern receptor mediated endocytosis and endocytic vesicle trafficking to specific subcellular compartments lie beyond the scope of this introduction.

1.1.8.3. ADCC

Antibody-directed cellular cytotoxicity (ADCC), a process whereby antibody coated cells are destroyed, is mediated by Fc receptors. This process is most commonly mediated by natural killer cells (NK cells) via FcγRIIIa. Monocytes and macrophages are also capable of mediating ADCC through FcγRI (Fanger *et al.*, 1989). This response is

important in cancer surveillance and the clearance of virus infected cells (Valerius *et al.*, 1993, Ely *et al.*, 1996, van de Winkel & Capel, 1993).

1.1.8.4. Degranulation and the respiratory burst

Fc receptors on phagocytic cells, including monocytes, macrophages and neutrophils, can activate a number of mechanisms for destroying internalised pathogens. These include the release of proteases and other anti-bacterial proteins from preformed granules into the phagosome through a process known as degranulation. Further, Fc receptors can activate the NADPH oxidase system to produce highly toxic reactive oxygen species (ROIs), important in the killing of pathogens. Similarly, Fc receptors can induce the production of iNOS (inducible nitric oxide synthase) to produce potent anti-microbial reactive nitrogen intermediates (reviewed in Daeron, 1997a).

The process of degranulation is also important in the release of inflammatory mediators. This is most studied for the activation of mast cells by FcεRI. Here receptor aggregation induces the release of pre-formed mediators, such as histamine, from the cell (Metzger, 1999).

1.1.8.5. Induction of cytokine gene transcription

Fc receptors play an important role in coordinating the immune response by inducing the transcription and secretion of cytokines (Daeron, 1997a). For example, stimulation of Fcγ receptors on monocytes and macrophages leads to the synthesis of proinflammatory cytokines such as IL-1, IL-6, and TNF-α. Fcγ receptors have also been linked to the synthesis of the anti-inflammatory cytokine, IL-10 (Sutterwala *et al.*, 1998) and the neutrophil chemoattractant IL-8 (Marsh *et al.*, 1995).

1.2. Fc Receptor Signalling

The multiple signal transduction pathways activated by different Fc receptors and the B-cell and T-cell antigen receptors show remarkable similarity. Thus, understanding of Fc and antigen receptor signalling has moved forward in parallel. For this reason, this section deals largely with signalling pathways common to both antigen and Fc receptors. Due to the complex interplay between distinct signalling pathways it is important not to consider them in isolation. Rather, signalling should be considered as a dynamic circuit with multiple independent events being integrated to give a concerted response. Here, the major signalling pathways activated by both Fc and antigen receptors are discussed.

1.2.1. Initiation of Fc Receptor Signalling

1.2.1.1. The ITAM motif

The initiation of signal transduction by the structurally and functionally diverse immune receptors as the TCR, BCR and Fc receptors is mediated through strikingly similar mechanisms. One common feature of all these receptors is that, unlike the growth factor receptors, they lack intrinsic enzymic activity within their cytoplasmic domains. The breakthrough in this field came in 1989 with the recognition, by Micheal Reth, of a common sequence motif within the cytoplasmic tails of many components of antigen and Fc receptor complexes (Reth, 1989). This motif, which was known by diverse names such as ARAM, TAM, and the Reth motif, has generally come to be known as the immunoreceptor tyrosine based activation motif (ITAM), has revolutionised the field of immune receptor signalling (Cambier *et al.*, 1995, Cambier, 1995). The ITAM is defined by 6 conserved amino acids generally spaced over 18 amino acids with the consensus, (D/E)XXYXX(L/I)X_7YXX(I/L), where X is any amino acid. This motif is found in other membrane proteins, including the CD3 and ζ-chains of the TCR, the α and β chains of the BCR, and the γ-chain found associated with multiple Fc receptors. An atypical ITAM motif is also found in the tail of FcγRIIa where the spacing between the key YXX(I/L) repeats is increased from 7 to 12 amino acids (Van den Herik-Oudijk *et al.*, 1995b,

Cambier, 1995). Table 1.2 shows an alignment of a number of key ITAM containing sequences.

1.2.1.2. ITAMs mediate signal transduction

Following the recognition of the ITAM motif a number of independent groups demonstrated, using chimeric receptor constructs, that the ITAM motif was necessary and sufficient to initiate signal transduction (review in Cambier, 1995). It was also demonstrated that phosphorylation of the two tyrosines within the ITAM motif was critical for function. This, along with the observation that immune receptors recruit and activate Src (Section 1.2.2.1) and Syk (Section 1.2.2.2) family tyrosine kinases has led to a conserved model for immune receptor mediated signal transduction (Cambier & Johnson, 1995, Johnson *et al.*, 1995, Agarwal, Salem & Robbins, 1993).

Table 1.2 Immune receptor ITAM sequences

Receptor/Subunit	ITAM sequence	Residues between YxxL/I motifs
γ-chain *	**D**GV**Y**TG**L**STRNGET**Y**ET**L**	7
FcγRIIa	**D**GG**Y**MT**L**NPRAPTDDDKNI**Y**LT**I**	11
FcεRI β-chain	**D**RV**Y**EE**L**NIYSAT**Y**SE**L**	6
BCR α-chain	ENL**Y**EG**L**NLDDCSM**Y**EDI	7
BCR β-chain	**D**HT**Y**EG**L**DIDQTAT**Y**EDI	7
Consensus	**D/E** XX **Y** XX **L** X(7-11) **Y** XX **L/I**	7-11

* γ-chain associates with FcγRI, FcγRIII, FcαRI and FcεRI

1.2.1.3. Immune receptors are activated by receptor clustering

Immune receptors, including the BCR, and Fc receptors propagate signals in response to receptor ligation or clustering. In the case of Fcγ receptors such receptor clustering occurs when cells, such as macrophages, encounter IgG immune complexes. In the case of the BCR, such ligation occurs when the B-cell encounters specific multivalent antigen. In the current model, partially active Src family kinases associated with resting ITAMs are activated on receptor clustering, leading to phosphorylation of the two key ITAM tyrosines. The phosphorylated ITAMs, then recruit and activate further Src kinases and Syk family kinases through SH2 domain interactions. This results in a cascade of tyrosine phosphorylation events which initiate multiple signalling pathways and subsequent effector functions (Figure 1.3) (Cambier, 1995, Agarwal *et al.*, 1993).

1.2.1.4. Lipid rafts provide a microenvironment for immune receptor activation

Recent advances in membrane biology have led to the identification of sphingolipid and cholesterol rich microdomains on the surface of immune cells. These microdomains, termed rafts, are enriched for specific signalling molecules, including the acylated Src family kinases, GPI anchored membrane proteins and Gα subunits of heterotrimeric G-proteins (Langlet *et al.*, 2000). Following ligation, many immune receptors, including to date the BCR, TCR, FcεRI and FcαRI relocate to these microdomains. Further, confocal microscopy has demonstrated that tyrosine phosphorylation events, and recruitment of many signalling components, occur specifically in these microdomains. These lipid rafts therefore appear to provide a microenvironment for receptor mediated activation of tyrosine kinases and subsequent signalling events (reviewed in Langlet *et al.*, 2000).

1.2.2. Tyrosine kinases

Many of the earliest signal transduction responses of Fc and antigen receptors are dependent on the activation of protein tyrosine kinases. The focus of this section is to discuss the role of Src and Syk family tyrosine kinases in the initiation of Fc and antigen receptor signalling. The role of the Tec family tyrosine kinases as effectors of PI 3-kinase will be discussed separately in section 1.2.3.4.

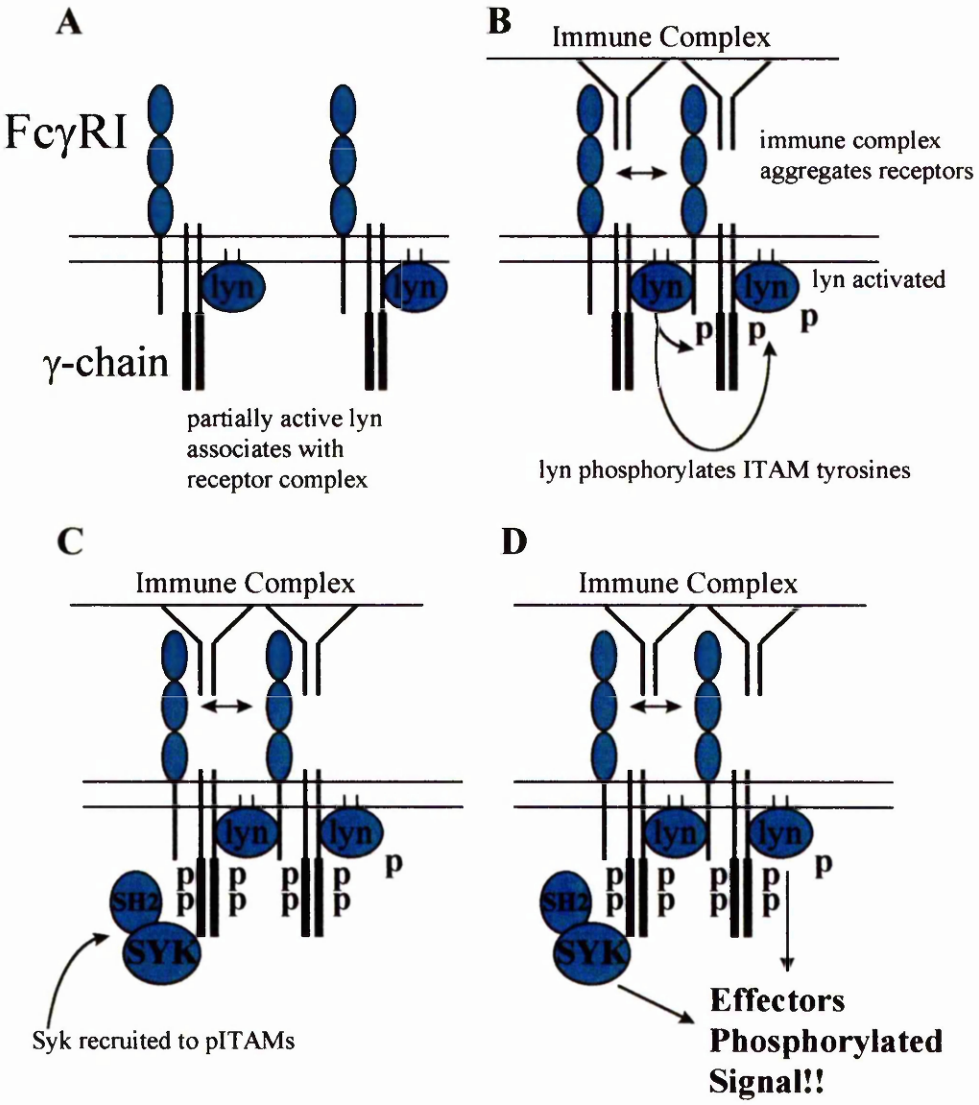


Figure 1.3. A model for Fc receptor activation. This model is described in section 1.2.1.3.

1.2.2.1. Src family tyrosine kinases

The src family of tyrosine kinases (Src, Lyn, Fyn, Lck, Blk, Fgr, Hck and Yes) are expressed widely though with some degree of cell-type specificity (Bolen, 1995). These kinases are all membrane associated by virtue of post translational N-terminal myristoylation and also, in most cases, palmitylation. These kinases contain an SH2 domain and an SH3 domain to allow association with tyrosine phosphorylated proteins, and proline rich regions, respectively. The localisation of these kinases at the plasma membrane, along with considerable evidence that these kinases are pre-associated with ITAM bearing receptors has placed them as likely candidates for mediating ITAM phosphorylation. Thus, Lck pre-associates with the TCR (Kane, Lin & Weiss, 2000), while Lyn preassociates with the Fc receptor γ-chain (Duchemin & Anderson, 1997) and the BCR (Clark *et al.*, 1992). Recent studies using transgenic mice lacking specific Src kinases has indicated the importance of these kinases in immune receptor function (Fitzer-Attas *et al.*, 2000). Of particular relevance, a dominant role for Lyn in Fcγ receptor mediated γ-chain ITAM phosphorylation and Syk activation has been reported. These studies have also revealed significant redundancy of function between family members of Src kinases (Fitzer-Attas *et al.*, 2000).

1.2.2.2. Syk family kinases

The Syk family of tyrosine kinases comprises Syk and ZAP-70 (Turner *et al.*, 2000). Syk is widely expressed cells of haematopoietic origin while ZAP-70 is restricted primarily to T-cells and NK cells. Syk family kinases are cytosolic proteins which contain a C-terminal kinase domain and two tandem SH2 domains that bind phosphorylated ITAMs (Cambier, 1995, Agarwal *et al.*, 1993). In addition, Syk contains multiple inter-domain tyrosines which act as docking sites for multiple signalling proteins, such as PLCγ and Vav, which are likely to be substrates for phosphorylation. The essential roles of Syk in mediating Fc receptor functions has been demonstrated using targeted disruption of the Syk gene in mice (Crowley *et al.*, 1997). These mice show an embryonic lethal phenotype. Almost all signalling and effector pathways activated by Fcγ receptors, including phagocytosis, ADCC, PLCγ activation and calcium mobilisation,

have been shown to be diminished or absent in cells derived from these mice. Defects in BCR, TCR and FcεRI function have also been observed. Surprisingly, Fcγ receptor ligation in Syk deficient mouse macrophages does not induce γ-chain ITAM phosphorylation. Conversely, FcεRI induced γ-chain phosphorylation in mast cells was unaffected in Syk deficient cells (reviewed in Turner *et al.*, 2000).

1.2.3. PI 3-kinase dependent signalling

Phosphoinositide-3 kinases (PI 3-kinases) play an important role in the generation of lipid second messengers critical for the transduction of multiple signalling pathways (Toker & Cantley, 1997, Leevers, Vanhaesebroeck & Waterfield, 1999). PI 3-kinases phosphorylate phosphatidylinositol (PI) lipids at the D3 position on the inositol ring to generate PI(3)P, PI (3,4)P_2 and PI (3,4,5)P_3 (depending on the substrate phosphorylated). In resting cells, 3' phosphoinositides are generally absent, and accumulate rapidly following diverse agonist stimulation (including Fc and antigen receptor engagement). Further, inhibition of PI 3-kinase activity results in inhibition of multiple cellular responses indicating a critical role for these enzymes in signal transduction (Figure 1.4).

1.2.3.1. Classes of PI 3-kinase

There are three classes of PI 3-kinase, being defined by their substrate specificities (Toker & Cantley, 1997). Class I PI 3-kinases phosphorylate phosphatidyl inositol (PI), PI(4)P, PI(4,5)P_2 to generate PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 respectively. Class I PI 3-kinases can be further subdivided into Class IA and Class IB. Class IA PI 3-kinases comprise a p110 catalytic subunit (p110α, p110β or p110δ) and a regulatory SH2/SH3 containing p85 family (p85α, p85β, p55α or p55 PIK) adapter subunit. The p85 adapter proteins facilitate binding to other proteins via SH2 and SH3 domain interactions. These isoforms of PI 3-kinase are generally activated by tyrosine kinase coupled receptors, including antigen receptors, Fc receptors and receptor tyrosine kinases (e.g. Insulin receptor). Class IB isoforms of PI 3-kinase comprise a p110γ catalytic subunit. Activity of p110γ is facilitated by interaction with the βγ subunits of heterotrimeric G-proteins, and are

thus activated by G-protein coupled receptors. Class II PI 3-kinases phosphorylate PI and PI(4)P but not PI(4,5)P_2 and Class III PI 3-kinases phosphorylate only PI.

1.2.3.2. Targets of PI 3-kinase signalling

It is now widely recognised that the products of PI 3-kinase, and in particular PIP_2 and PIP_3, are important regulated membrane localisation signals (Czech, 2000, Leevers *et al.*, 1999). Of particular relevance to membrane targeting is the pleckstrin homology (PH) domain which mediates both protein:protein and protein:lipid interactions. Indeed, several PH domain containing proteins have been shown to selectively bind PIP_2 and/or PIP_3, including small G-protein exchange factors Sos and Vav (section 1.2.5.2), the Akt/PKB Ser/Thr kinase (section 1.2.3.3), PLCγ isoforms (Falasca *et al.*, 1998) and the Tec family of tyrosine kinases (section 1.2.3.4). However, other domains, including SH2 and protein tyrosine binding (PTB) domains have also been shown to bind to PIP_2 and/or PIP_3 indicating a further level of complexity (Figure 1.4) (Toker & Cantley, 1997).

1.2.3.3. Akt/PKB

The serine/threonine protein kinase Akt, also known as protein kinase B (PKB) has emerged as a major target of PI 3-kinase signalling (Franke *et al.*, 1995, Burgering & Coffer, 1995). Akt has a PH domain that binds both PIP_2 and PIP_3. Some evidence suggests that binding of phosphoinositides to the PH domain not only recruits PKB to the plasma membrane, but also plays a role in its activation by inducing conformational changes (Franke *et al.*, 1997). For activation, Akt requires phosphorylation on both Thr308 and Ser473. The kinase responsible for phosphorylating Thr308, termed PDK1 (phosphoinositide dependent kinase 1), also contains a PH domain. PIP_3 has also been shown to be required to recruit PDK1 to the plasma membrane for activation (Alessi *et al.*, 1997). The kinase responsible for Akt phosphorylation on Ser473 (termed PDK2) remains to be identified.

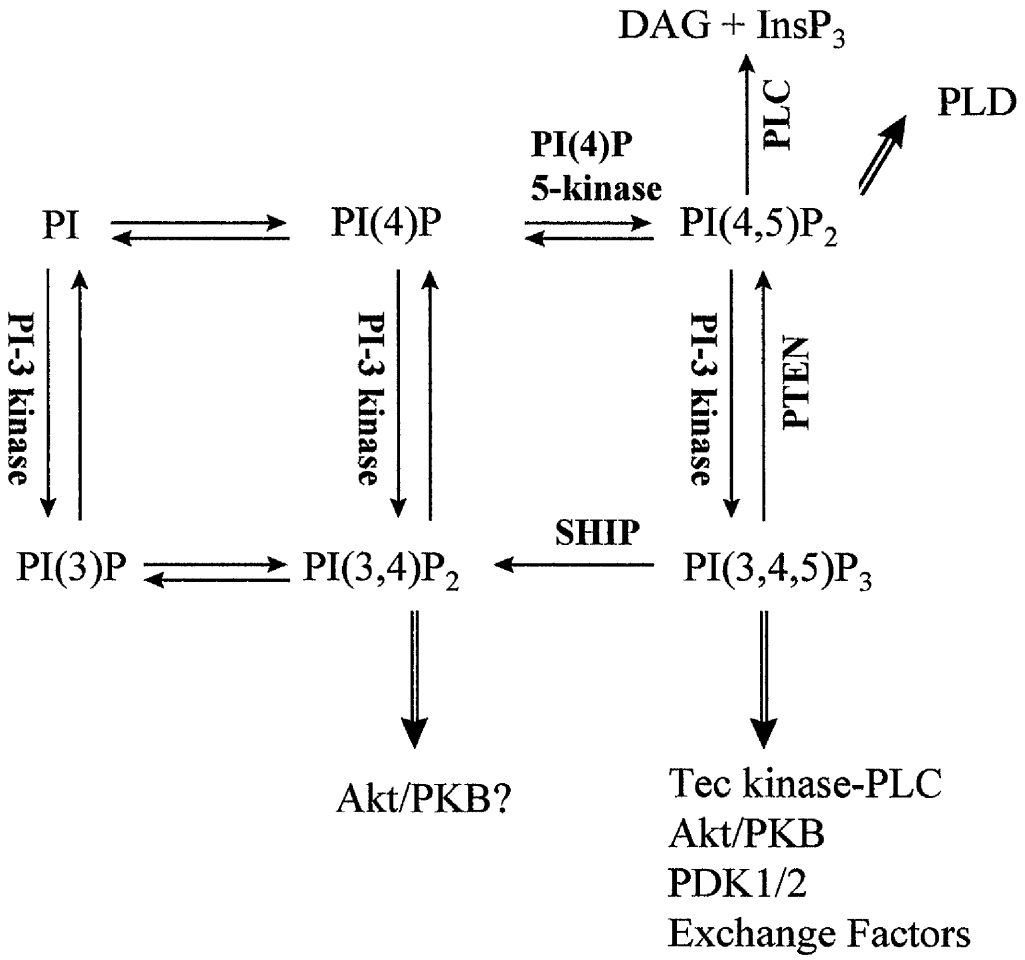


Figure 1.4. An overview of phosphoinositide signalling. Selected enzymes synthesising various phosphoinositides are indicated. Effector pathways are also indicated where pertinent to this thesis.

Akt has been implicated in the prevention of apoptosis by phosphorylating a range of proteins including Bad and caspase-9. A role for Akt in promoting cell cycle progression has also been proposed. Interestingly, Akt also phosphorylates glycogen synthase kinase-3 (GSK-3) which has been proposed to regulate the subcellular localisation of the calcium dependent transcription factor, NF-AT_C. (Gold *et al.*, 1999, Cross *et al.*, 1995). GSK-3 is constitutively active in cells and phosphorylates NF-AT_C, resulting in its rapid export from the nucleus. By inhibiting GSK-3, Akt may allow NF-AT_C to accumulate in the nucleus in response to calcium influx and thus promote transcription. In addition, recent studies have shown that PKB can positively regulate the transcription factor NFκB (Scheid & Woodgett, 2000, Ozes *et al.*, 1999, Romashkova & Makarov, 1999). Here, PKB appears to promote activation of IKK, the upstream kinase which phosphorylates the inhibitory component of the NFκB complex, IκB (Scheid & Woodgett, 2000). Once phosphorylated, IκB is degraded, freeing NFκB which can then translocate to the nucleus. These transcription factors, NF-AT_C and NFκB are of particular interest as they are thought to play important roles in the regulation of cytokine gene transcription. Recent reports have indicated that PKB is activated by Fcγ receptors and G-protein coupled receptors in macrophages and by the BCR in B-cells (Tilton *et al.*, 1997). Further, Akt is a target of FcγRIIb/SHIP mediated inhibition (Jacob *et al.*, 1999) (discussed in section 1.2.8.12).

1.2.3.4. Tec family tyrosine kinases

The Tec family of protein tyrosine kinases, which includes Btk, Tec, Bmx and Itk, have emerged as an important group of non-receptor protein tyrosine kinases (Schaeffer & Schwartzberg, 2000, Bolen, 1995). All four kinase have closely homologous structures which include an N-terminal PH domain followed SH2, SH3 and kinase domains. In the case of Btk, the prototype Tec kinase, interaction of its PH domain with PIP_3 is sufficient to induce its recruitment and activation through autophosphorylation and phosphorylation by Src and/or Syk kinase family members. The importance of the Btk PH domain is clear, as inactivating mutations in the Btk PH domain have been found to confer the phenotype of Bruton's disease or X-linked agammaglobulinaemia (XLA), a

severe immune deficiency disease of humans. There is a naturally occuring mutant in the mouse, X-linked immunodefficiency (Xid), that generates a phenotype similar to Bruton's diease in man. The mutation in the Xid mouse is in the PH domain of Btk. The dependence of these kinases on PIP_3 places them as important downstream effectors of PI 3-kinase activation. The downstream targets of Tec family kinases include apoptotic pathways, JNK (c-jun N-terminal kinase) and PLCγ.

1.2.3.5. Tec family kinases are required for PLCγ activation

Of particular importance, Tec family kinase activation appears to be essential for PLCγ activation (discussed in section 1.2.4.3) by antigen receptors (Fluckiger *et al.*, 1998). Studies in B-cells have demonstrated that participation in PLCγ activation is a property of all Tec family members and that their role is independent from that of Src and Syk family kinases. Further, this PI 3-kinase/Tec/PLCγ pathway has been shown to be a central target for FcγRIIb/SHIP mediated inhibition (Scharenberg *et al.*, 1998, Bolland *et al.*, 1998) (section 1.2.8.9).

1.2.4. Signalling pathways leading to the mobilisation of calcium

Changes in cytosolic calcium is a major component of signalling by many immune receptors. Calcium levels can be elevated following release from intracellular stores and by calcium entry. The central event leading to calcium store release in response to receptor stimulation is the activation of PLCγ to generate inositol 1,4,5-trisphosphate ($InsP_3$) (Berridge, 1993, Tsien & Tsien, 1990, Putney, 1986). $InsP_3$ induces the rapid release of calcium from internal stores through the binding of $InsP_3$ to the $InsP_3$ receptor (Figure 1.5). This, in turn induces store-operated calcium influx through the cytoplasmic membrane calcium channels termed I_{CRAC} (calcium release activated current) (Hoth & Penner, 1992).

1.2.4.1. Calcium is a key regulator of cell activation

Elevated intracellular calcium is a constant feature of cell activation triggered by ITAM bearing receptors in a number of cell types. Receptor induced calcium oscillations

regulates a number of key signalling events, including PKC activation (see section 1.2.4.4) and gene transcription (Figure 1.5). Increases in calcium also regulate calmodulin dependent events including the activation of Ca^{++}/calmodulin dependent kinase (De Koninck & Schulman, 1998) and the calmodulin dependent serine threonine phosphatase, calcineurin. Elegant studies have demonstrated that calcium oscilations regulate the nuclear localisation and activity of the transcription factors NFAT (regulated by calcineurin) and NFκB, which play a central role in antigen and Fc receptor mediated gene transcription (Figure 1.5) (Dolmetsch, Xu & Lewis, 1998, Hu *et al.*, 1999, Li *et al.*, 1998)

1.2.4.2. PLC

The phosphoinositide phospholipase C (PLC) family are enzymes which specifically hydrolyse phosphatidylinositol-4,5-bisphosphate $PI(4,5)P_2$ to generate the second messengers inositol trisphosphate ($InsP_3$) and diacylglycerol (DAG) (Lee & Rhee, 1995). There are three homologous groups of PLC (β, γ and δ), all of which contain PH domains. PLCγ also contains tandem SH2 domains and an SH3 domain. In tyrosine kinase dependent signalling, including most immune receptors, it is the PLCγ isoforms (PLCγ1 or PLCγ2) which are activated.

1.2.4.3. The mechanism of PLCγ activation - the role of adapter protein complexes

The molecular mechanisms underlying PLCγ activation in response to ligation of the TCR and the BCR have been largely defined. In the case of the TCR, the adapter proteins, LAT (Linker for the Activation of T-cells) and SLP-76 (SH2 domain containing leukocyte protein of 76 kDa), have been shown to play a key role in this process (Finco *et al.*, 1998, Yablonski *et al.*, 1998, Myung, Boerthe & Koretzky, 2000). These proteins are heavily tyrosine phosphorylated in response to TCR ligation. Here, activation of the Src family kinase, Lck, and the Syk analogue, ZAP-70, induces phosphorylation of the membrane associated adapter LAT. LAT in turn recruits PLCγ1 to the plasma membrane. However, for activity, PLCγ must be phosphorylated by both Syk and Tec family kinases (ZAP-70 and Itk for the TCR). The Tec family kinase appears to require the second adapter protein, SLP-76, to facilitate PLCγ1 activation. SLP-76 is recruited to

LAT via the Grb2 homologue, Gads (Liu *et al.*, 1999). Thus, SLP-76, is recruited to LAT-PLCγ1 complexes, where it plays an intermediary between Itk and PLCγ1 to allow activation. Interestingly, no equivalent to LAT has been identified in B-cells. However, the B-cell specific adapter protein, BLNK (also known as SLP-65), appears to play the role of intermediary between PLCγ2 and the Tec family kinase, Btk, in an analagous role to SLP-76 in T-cells (Ishiai *et al.*, 1999, Wienands *et al.*, 1998, Wong *et al.*, 2000, Kurosaki & Tsukada, 2000). As discussed in section 1.2.3.4, the recruitment of Tec family kinases to the plasma membrane has an additional requirement for PH domain binding to PIP_3 (Fluckiger *et al.*, 1998). Likewise, the PH domain of PLCγ isoforms, which also binds PIP_3, is essential for activity (Falasca *et al.*, 1998). Similar mechanisms are likely to govern Fcγ receptor mediated PLCγ activation. Indeed, both Fcγ receptors in monocytes have been reported to induce the tyrosine phosphorylation of the SLP-76 adapter protein and its association with other tyrosine kinase substrates (Chu *et al.*, 1998).

1.2.4.4. Calcium and DAG regulate PKC activation

The protein kinase C (PKC) family of serine/threonine kinase can be divided into three subgroups on the basis of their co-factor regulation (Nishizuka, 1988, Nishizuka, 1992, Parekh, Ziegler & Parker, 2000, Dekker & Parker, 1994). Thus, conventional PKC isoforms (PKCα, β1, β2 and γ) are dependent on calcium and DAG for activation. Novel PKC isoforms (δ, ε, η, θ,and μ) require DAG but not calcium, while the atypical PKC isoforms (ζ) require neither calcium or DAG. PLCγ activation regulates PKC isoform activation by generating DAG and mobilising calcium. It is important to point out that phospholipase D (PLD) provides an alternative pathway to DAG (see section 1.2.7) though it is unclear whether PLD derived DAG is capable of regulating PKC activity. Activation of PKC is well established as being essential for phagocytosis in myeloid cells (Zheleznyak & Brown, 1992). PKC activation also plays a role in the regulation of the MAP kinase cascades (section 1.2.6.3). The role of different PKC isoforms in FcγRI signalling is discussed in section 1.4.5.

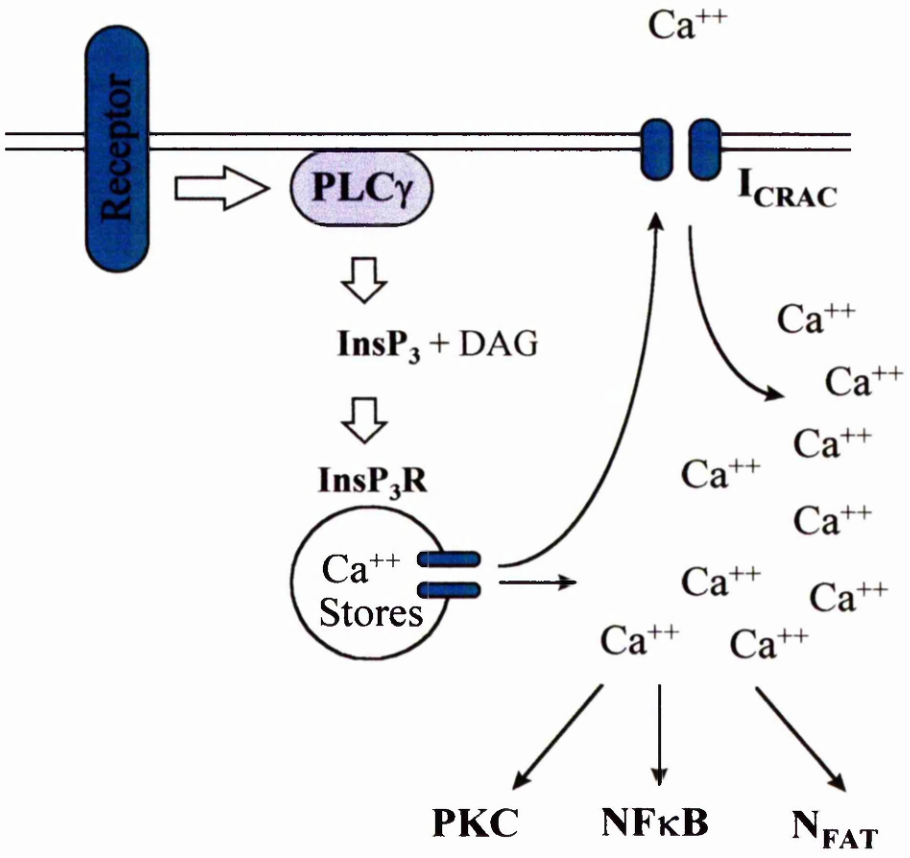


Figure 1.5. An overview of PLCγ mediated calcium signalling. PLCγ activation catalyses the production of $InsP_3$ from PIP_2. $InsP_3$ induces the rapid release of calcium from internal stores through binding to the $InsP_3$ receptor. This in turn induces store-operated calcium influx through the cytoplasmic membrane calcium channels (I_{CRAC}).

1.2.4.5. PLCγ/InsP$_3$ independent calcium signalling

An alternative InsP$_3$ independent pathway to calcium store release has also been described by us and others (Melendez *et al.*, 1998b, Choi, Kim & Kinet, 1996). Here, tyrosine kinase activation leads to the PI 3-kinase dependent activation of phospholipase D and sphingosine kinase (Melendez *et al.*, 1998b). Calcium store release here is thought to be mediated by the sphingolipid-gated calcium channel situated in the endoplasmic reticulum, termed the SCaMPER (Sphingolipid Ca^{++} release Mediating Protein of Endoplasmic Reticulum) receptor (Mao *et al.*, 1996). Due to the particular relevance of this pathway to FcγRI signalling, it is covered in more detail in section 1.4.3.

1.2.5. Small GTPase coupled pathways

The mammalian superfamily of small GTPases includes in excess of 80 members of which the Ras proteins are the prototypes. Small GTPases cycle between inactive GDP bound and active GTP bound states and, thus, function as signal relays linking membrane receptors to signal transduction pathways (Rommel & Hafen, 1998, Henning & Cantrell, 1998, Scita *et al.*, 2000, Genot & Cantrell, 2000). Activated GTPases bind to a wide variety of effector molecules to initiate multiple distinct signalling pathways. As many of these GTPases bind multiple distinct effector molecules, they act as versatile cellular switches. Here, discussion will be limited primarily to the Ras and Rho (including Rac1, cdc42 and Rho) families of small GTPases. Discussion will also be limited to the roles of these GTPases in mediating a subset of effector functions relevant to Fc receptor biology. Firstly, the role of Ras and Rac1 (a Rho family member) GTPases in mediating immune receptor activation of MAP kinase pathways will be covered. The involvement of the Rho GTPases in mediating phagocytosis will also be discussed due to its particular relevance to Fc receptors. The involvement of the ARF family GTPases in the regulation of PLD and membrane trafficking will be discussed separately in section 1.2.7.1.

1.2.5.1. Small GTPase activity is governed by GEFs, GAPs and GDIs

Guanine nucleotide binding by small GTPases is governed by the activity of guanine nucleotide exchange factors (GEFs) which promote the transition from a GDP bound inactive state, to the GTP bound active state. This effect is balanced by the activity of GTPase activating proteins (GAPs) which stimulate GTPase activity thereby promoting the hydrolysis of GTP and return these small GTPases to the GDP bound inactive state. A third class of proteins, the guanine nucleotide dissociation inhibitors (GDIs) regulate GTPase activity by inhibiting the release of GDP. Thus the activity of these GTPases is governed by the balanced activity of GEFs, GAPs and GDIs (Henning & Cantrell, 1998, Scita *et al.*, 2000).

1.2.5.2. Ras and Rac1 activation by immune receptors: Adapter protein complexes recruit GEFs

Analagous to receptor tyrosine kinases, Fc receptor and antigen receptors induce recruitment of cytosolic exchange factors to the plasma membrane, where guanine nucleotide exchange can take place. Recruitment of these exchange factors involves the formation of adapter protein scaffolds. Thus, Ras activation appears to involve recruitment of the Ras exchange factor, Sos to the plasma membrane. Here, the adapter protein Grb-2 binds Sos via its SH3 domain. The SH2 domain of Grb-2 then allows recruitment of Sos to membrane proximal signalling complexes where activation of Ras tales place (Gale *et al.*, 1993, Li *et al.*, 1993, Rozakis-Adcock *et al.*, 1993). In the cases of FcεRI and the TCR, this recruitment involves Grb-2 binding to the membrane anchored adapter, LAT (Finco *et al.*, 1998). In B-cells, which do not express LAT, the formation of Shc-Grb-2-Sos complexes appears to be important (Dambrosio, Hippen & Cambier, 1996).

In a similar mechanism, the exchange factor Vav, links the T-cell receptor and FcεRI to the Rho family members, Rac1, cdc42 and Rho (Teramoto *et al.*, 1997, Arudchandran *et al.*, 2000). In these systems, recruitment of Vav to the plasma membrane occurs through the SLP-76 adapter protein. The SH3 domain of SLP-76 binds to proline rich regions in Vav. Following receptor aggregation, SLP76 is recruited to the plasma membrane via indirect

interactions with LAT. Both Sos and Vav also have PH domains which bind the products of PI 3-kinase though the relative contributions of PH domain targeting and adapter protein interactions in the recruitment of these exchange factors is not clear.

1.2.5.3. Ras effector pathways in immune receptor signalling

Ras plays a central role in the proliferation, growth, differentiation and death of all mammalian cells. Ras also mediates cell specific functions by linking receptors to specific effector pathways (Rommel & Hafen, 1998, Genot & Cantrell, 2000). Its actions are mediated through binding to effector molecules. The two best known effectors of Ras are the protein kinase, Raf-1, and PI 3-kinase. The primary target of Raf-1 is the MEK/ERK pathway which is discussed in section 1.2.6.2. Activated Ras directly associates with and activates the p110 catalytic subunit of p85 dependent PI 3-kinase. This provides a second potential mechanism for linking immune receptors to PI 3-kinase dependent pathways. PI 3-kinase signalling by immune receptors is discussed in section 1.2.3.

A further critical target for Ras mediated signals are the NFAT (nuclear factor of activated T-cells) family of transcription factors. These transcription factors are important for receptor induced transcription of multiple cytokines. NFAT is activated by antigen receptors in B and T-cells as well as by FcεRI in mast cells and FcγRIII in NK cells. NFAT activation requires the co-ordinated interaction of receptor induced Ras activation and receptor activated calcium/calcineurin. The mechanism linking Ras to NFAT is not clear though an intermediary role for Rac has been proposed (Henning & Cantrell, 1998).

1.2.5.4. Effector pathways of Rho family GTPase

Like Ras, Rac mediates its effects through diverse signalling pathways (Aspenstrom, 1999). One key set of effectors activated in the Rac1 and cdc42 dependent pathways are the p38 and JNK families of MAP kinases (Zhang *et al.*, 1995, Coso *et al.*, 1995). A role for p21 activated kinase (PAK) in the regulation of kinase cascades leading to JNK and p38 MAP kinase pathways has been proposed. Both Rac and cdc42 interact with a

number of other kinases capable of activating MAP kinase cascades such as MEKK and MEKK4 (Aspenstrom, 1999).

Members of the Rho family of small GTPases co-ordinate cytoskeletal reorganisation induced by diverse stimuli. As phagocytosis, by definition, requires reorganisation of the actin based cytoskelleton (section 1.1.8.1), the involvement of Rho family GTPases has been investigated. Essential roles for the Rho family members, Rac1, cdc42 and Rho in phagocytosis have been established (Cox *et al.*, 1997, Hackam *et al.*, 1997). Interestingly, the use of C3 toxin to inhibit Fcγ receptor mediated Rho activation in macrophages was found to block not only phagocytosis, but also tyrosine phosphorylation and calcium signalling. This effect on tyrosine phosphorylation and calcium signalling is thought to result from a disruption of receptor-cytoskeleton interactions required for efficient receptor clustering (Hackam *et al.*, 1997).

Among the other targets for Rho family members are $p67_{PHOX}$, (phagocyte oxidase) PI(4)P 5-kinase and phospholipase D (PLD) (Aspenstrom, 1999). $P67_{PHOX}$ is a component of the NADPH oxidase complex, critical for the respiratory burst (section 1.1.8.4). $P67_{PHOX}$ is a potential substrate for phosphorylation by PAK, an effector for Rac and cdc42. PI(4)P 5-kinase is responsible for catalysing the production of $PI(4,5)P_2$, which is the substrate of PLCγ. $PI(4,5)P_2$ is also essential for receptor mediated activation of PLD. Regulation of PLD is discussed separately in section 1.2.7.1. Finally, a critical role for Rac in FcεRI mediated NFAT nuclear import has been demonstrated, though the mechanism for this action is not known (Turner *et al.*, 1998).

1.2.6. MAP kinases - background

The MAP kinases are a growing family of proline directed serine-threonine protein kinases which are activated by a wide variety of extracellular stimuli (Garrington & Johnson, 1999, Su & Karin, 1996). The MAPKs can be divided into a number of subgroups, which include the extracellular regulated kinases (ERKs), c-Jun N-terminal kinase (JNK) and the p38 group of kinases. The ERK family are the most widely studied of the MAP kinases due to their central role in mediating cell proliferation and

transformation (cancer). The p38 and JNK MAP kinases fall under the subgroup of stress activated protein kinases as they are rapidly activated in response to environmental stresses such as osmotic shock and ultra-violet irradiation. These kinases play key roles in stress response, apoptosis and inflammation (Paul *et al.*, 1997, Herlaar & Brown, 1999). Among the substrates of the MAP kinases are a wide variety of transcription factors. Thus, the kinases provide a link between extracellular stimuli and transcription. Numerous other cellular effector functions are also targets of MAP kinase regulation and those pertinent to Fc receptor function are discussed below.

1.2.6.1. Regulation of the MAP kinase pathways

The MAP kinase pathways are regulated by protein kinase cascades that are evolutionarily conserved from yeast to man (Garrington & Johnson, 1999). At the core of these cascades lies a three kinase module. The MAP kinases are all activated by dual phosphorylation of a common threonine-X-tyrosine motif (Table 1.3). This phosphorylation is mediated by distinct dual specificity MAP kinase kinases (MKKs). These kinases are in turn activated by serine /threonine phosphorylation by MAP kinase kinase kinases (MKKK, also known as MEKKs). This MEKK-MKK-MAPK model for activation is typified by the Raf-MEK-ERK cascade. The MKKs and MEKKs for the p38 and JNK pathways are indicated in figure 1.6.

In mammalian cells, MAP kinases form parallel signalling pathways often activated in response to the same stimulus. Thus, in immune receptor signalling, a wide variety of receptors, which include the BCR, FcεRI and FcγRs, have been reported to activate ERK, p38 and JNK (Hashimoto *et al.*, 1998, Ishizuka *et al.*, 1997, Ishizuka *et al.*, 1999, Rose *et al.*, 1997). The focus of the following section lies on the activation of MAP kinase cascades by tyrosine kinase linked receptors and, in particular, on their activation by antigen and Fc receptors.

Table 1.3 Dual phosphorylation sites within the MAP kinase sequences

MAP kinase	Dual Phosphorylation Site
ERK	**--TEY--**
p38	**--TGY--**
JNK	**--TPY--**

1.2.6.2. ERK is a downstream effector of Ras

The most documented MAPK activation pathway is the Ras-Raf-MEK-ERK pathway. Here, activated Ras recruits the serine/threonine kinase Raf-1 to the plasma membrane. Raf-1 then phosphorylates and stimulates the MAP kinase kinase, MEK (MEK1 or MEK2), which is responsible for the dual phosphorylation of ERK. This pathway is utilised by the TCR. Thus, TCR-mediated ERK activation can be inhibited by expression of dominant negative Ras and specific inhibitors of MEK (Izquierdo *et al.*, 1993). Likewise, the BCR induces Ras dependent ERK activation although an additional pathway dependent on PKC also appears to be involved (Gold *et al.*, 1992).

1.2.6.3. Ras independent ERK activation - a role for PKC

A number of stimuli have been shown to activate the ERK pathway independent of Ras activation. Thus, phorbol ester stimulation of fibroblasts, which activates PKC, induces ERK activation independent of Ras but dependent on Raf-1 (Ueda *et al.*, 1996). Further, MEK dependent but Raf-1 independent activation of ERK has also been reported in fibroblasts implying that PKCs exert regulatory control on ERK activation at multiple levels (Schonwasser *et al.*, 1998). Raf-1 has also been identified as a target of PLD

mediated signalling (Rizzo *et al.*, 1999). Here, phosphatidic acid (PA), a product of PLD, appears to be important for the translocation of Raf-1 to the plasma membrane. These Ras independent pathways are of particular interest as a recent report has suggested that Fcγ receptors in monocytes activate ERK independent of both Raf-1 and Ras (Sanchez-Mejorada & Rosales, 1998). Sanchez-Mejorada *et al* found that ERK activation by immune complex in both THP-1 and U937 cells was unaffected by dominant negative isoforms of Ras and Raf-1. They further demonstrate that Ras and Raf-1 independent activation of ERK by Fcγ receptors is coupled to NFκB transcription of cytokine genes. The mechanism linking FcγR to ERK activation is yet to be determined.

1.2.6.4. Regulation of JNK and p38

As described in section 1.2.6.1, p38 and JNK are also regulated by a central three component kinase cascade. The regulation of these cascades is, however, less well defined than for the ERK pathway. In mechanisms analogous to the Ras activation of ERK, the JNK and p38 MAP kinases have been shown to be downstream effectors of the Rac1 and cdc42 GTPases (Zhang *et al.*, 1995, Coso *et al.*, 1995, Hashimoto *et al.*, 1999). Here, activated GTPases initiate kinase cascades leading to the activation of p38 and JNK. p21 activated kinase (PAK) is a candidate effector of Rac1 leading to the activation of p38 and JNK. PAK is thought to phosphorylate JNK and p38 MEKK family members (Figure 1.6).

The BCR has been shown to mediate Rac1-dependent activation of both p38 and JNK. p38 appears to require PKC activation indicating that further levels of regulation exist (Hashimoto *et al.*, 1998). BCR induced JNK activation also appears to have additional dependence on calcium release; this activation may occur through a calcineurin dependent mechanism. Little is known concerning the mechanisms governing Fcγ receptor mediated activation of p38 and JNK.

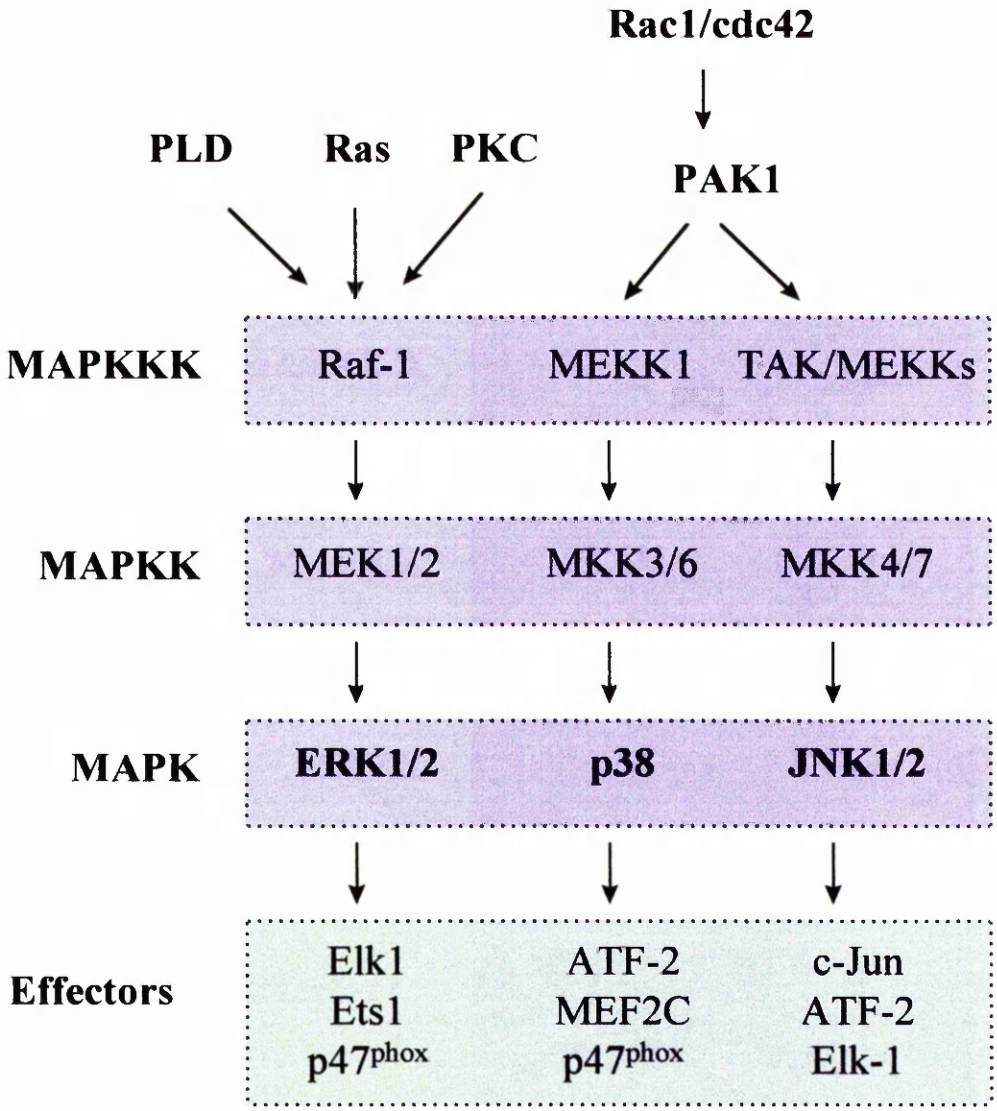


Figure 1.6. An overview of the MAP kinase cascades. These pathways are described in more detail in section 1.2.6.

1.2.6.5. MAP kinases regulate transcription factors

The MAP kinase cascades play a pivotal role in linking signal transduction pathways to transcriptional responses by phosphorylating, and thus modulating the activity of, transcription factors (Su & Karin, 1996, Garrington & Johnson, 1999). Each family of MAP kinases exhibits specificity for different subsets of transcription factors. Figure 1.6 shows some of the transcription factor targets of the different MAP kinases. The AP1 transcription factor is the prototype of transcription factors regulated by the MAP kinases. AP1 is composed of stable c-Jun and c-Fos dimers. C-Jun and c-Fos are early activation genes. Their expression is characteristically low in resting cells and is induced by extracellular signals. Thus, c-Jun transcription can be initiated by phosphorylation of the constitutively expressed MEF2C or c-Jun-ATF2 dimers. Similarly, phosphorylation of Elk1 can initiate c-Fos transcription. Further, phosphorylation of c-Jun by JNK MAP kinases greatly enhances its ability to activate transcription. As MEF2C, Elk1, ATF2 and c-Jun are targets for various MAP kinases, MAP kinase activation can induce AP1 transcription (Su & Karin, 1996).

The relevance of MAP kinase induced transcription in Fc receptor signalling has been firmly established. In murine macrophages, Fcγ receptor ligation induces increased AP-1 binding (Cote-Velez, Ortega & Ortega, 1999). Further, in human monocytes and NK cells, Fcγ receptors are coupled through ERK activation to the transcription of c-Fos and TNF-α (Trotta, Kanakaraj & Perussia, 1996). Studies using specific inhibitors of ERK have also indicated a role in mediating Fcγ receptor induced NFκB activation in monocytes (Sanchez-Mejorada & Rosales, 1998).

1.2.6.6. MAP kinases synergise with other signals to induce transcription.

Interestingly, AP1 transcription factors act in concert with NFAT transcription factors in the nucleus to induce transcription (Genot & Cantrell, 2000, Turner *et al.*, 1998). This indicates crosstalk between the MAPK pathways and calcium:calcineurin signalling at the transcriptional level. Other studies have placed ERK upstream of NFκB in Fcγ receptor

signalling, though the mechanism for this regulation remains unclear (Sanchez-Mejorada & Rosales, 1998). Other factors including calcium mobilisation and PKB activation play important roles in NFκB activation indicating that multiple signalling pathways converge at the level of transcription.

MAP kinase are also coupled to transcription independent effector functions. For example, ERK and p38 have also been implicated in Fcγ receptor stimulation of cytosolic phospholipase A (PLA) and the activation of the NADPH oxidase system (Herlaar & Brown, 1999, Hazan-Halevy & Levy, 2000, Hazan-Halevy, Seger & Levy, 2000).

1.2.7. PLD

The phospholipase D (PLD) family catalyse the hydrolysis of membrane phospholipids, in a substrate specific manner (Exton, 1994, Exton, 1999). Phosphatidylcholine PLD (PC-PLD) hydolyses PC to generate phosphatidic acid (PA) and choline. The downstream targets of phosphatidic acid are not well defined. However, PA has been implicated as a lipid second messenger, regulating diverse signalling molecules, including Raf-1 (Rizzo *et al.*, 1999). Alternatively, phosphatidic acid can be converted by PA phosphohydrolase to diacylglycerol (DAG) which may also act as a second messenger to activate, for example, novel PKCs (see section 1.2.4.4). PLD appears to play a central role in mediating membrane trafficking in a wide variety of cells (Melendez *et al.*, 1998b, Roth *et al.*, 1999). Recently, a role for PLD in the regulation of Fcγ receptor phagocytosis has been demonstrated, although the mechanism for this regulation remains unresolved (Kusner, Hall & Schlesinger, 1996, Kusner, Hall & Jackson, 1999, Lennartz, 1999). In monocytes and neutrophils, a role for PLD in mediating the respiratory burst has also been reported (Exton, 1997).

1.2.7.1. Regulation of PLD

PLD is activated by a wide variety of stimuli including tyrosine kinase and heterotrimeric G-protein coupled receptors. Two are two known PLD isoforms, PLD1 and PLD2. These two enzymes are regulated diffferently. Both are activated by $PI(4,5)P_2$ (Pertile *et*

al., 1995). PLD1 is regulated through various additional mechanisms, though the small GTPases, Rho and Arf, and PKC appear to play dominant roles (Exton, 1997, Exton, 1999, Hammond *et al.*, 1995). The regulation of PC-PLD activation by FcγRI activity is discussed further in section 1.4.3.

1.2.8. Inhibitory Signalling

As knowledge of the pathways activated by ITAM bearing immune receptors has increased it has also become apparent that the activating signals sent by one receptor can be countered by signals sent from another 'inhibitory' receptor. Studies on the low affinity IgG receptor, FcγRIIb, long known for its inhibitory effects on B cell activation, led to the recognition of the ITIM motif (Amigorena *et al.*, 1992a, Muta *et al.*, 1994a). The subsequent identification of the same motif within the cytoplasmic tails of many immune receptors, including the NK cell killer inhibitory receptors (KIRs), the T-cell 'off switch' CTLA-4 and the BCR associated CD22 has led to an explosion in the field of inhibitory signalling (Daeron, 1996, Unkeless & Jin, 1997). The current consensus sequence for the ITIM is, (I/V)XYXXL, where X is any amino acid. This is reminiscent of the ITAM sequence, (D/E)XXYXX(L/I)X_7YXX(I/L), although the characteristic double YXXL motif found in the ITAM is replaced by a single YXXL motif. Two fundamentally distinct inhibitory mechanisms governing ITIM mediated inhibition have now emerged (Gupta *et al.*, 1997, Scharenberg & Kinet, 1996). The first, typified by the KIRs, involves recruitment of protein tyrosine phosphatases to counter tyrosine kinase induced protein phosphorylation and activation (Long *et al.*, 1997, Burshtyn & Long, 1997). The second mechanism involves the recruitment of the inositol 5'phosphatase, SHIP, to FcγRIIb (Ono *et al.*, 1996). Here, KIR inhibition of NK cell activity and FcγRIIb inhibition of B-cell and mast cell activation will be described as prototypical inhibitory receptors.

1.2.8.1. Killer inhibitory receptors recruit SHP-1 to inhibit NK cell activity.

A number of NK cell surface receptors, including FcγRIII, are able to mediate targte cell killng through cell lysis. When an NK cell encounters a target cell, it recognises and kills it

if it does not express MHC (major histocompatability complex) class I molecules. For example, NK cells target IgG bound cells for lysis via FcγRIII (See ADCC - section 1.1.8.3). If, however, the cells also express MHC class I molecules, which are the ligands for KIRs, cells are recognised as 'self' and are protected from lysis (Burshtyn & Long, 1997, Long *et al.*, 1997, Vely *et al.*, 1996). Here, FcγRIII mediates the cell killing by activating tyrosine kinase dependent signalling cascades through the ITAM bearing γ-chain. Co-activation of KIRs induces the tyrosine phosphorylation of the ITIM tyrosine in the KIR cytoplasmic tail. The phosphorylated ITIM acts as a docking site for the tyrosine phosphatase, SHP-1, which blocks proximal tyrosine kinase dependent activation. A whole host of receptors appear to utilize this or similar mechanisms, including CD22 and CD72 in B-cells, CTLA-4 in T-cells, and the family of Leukocyte Inhibitory Receptors (LIRs) (Daeron, 1996, Unkeless & Jin, 1997, Vely *et al.*, 1997).

1.2.8.2. SHP1 and SHP-2

The SH2 containing phosphatases, SHP-1 and SHP-2, comprise tandem SH2 domains, responsible for ITIM binding, and a phosphatase domain (Frearson & Alexander, 1997). SHP-2 is ubiquitously expressed, while SHP-1 is largely limited in expression to haematopoietic cells. An inhibitory role for SHP-1 is supported by the discovery that motheaten (me/me) mice, which show severe immunodefficiency, possess an inactivating mutation in the SHP-1 gene. The pathology of these mice results from marked overexpansion and overactivation of multiple haematopoietic cells. SHP-1 is now widely accepted as a negative regulator of many immune receptors, including antigen and Fc receptors (Frearson & Alexander, 1997). SHP-2 is documented as having both positive and negative effects on signalling. The inhibitory properties of SHP-2 are exemplified by its recruitment to the phosphorylated ITIM of CTLA-4 to negatively regulate T-cells (Burshtyn & Long, 1997).

1.2.8.3. FcγRIIb recruits SHIP to inhibit BCR and antigen receptor signalling

FcγRIIb is now widely accepted to inhibit the responses of a number of immune receptors, including the BCR on B-cells and FcεRI and FcγRIII on murine mast cells

(Ono *et al.*, 1996, Ujike *et al.*, 1999). Unlike the KIRs discussed above (section 1.2.8.2), FcγRIIb does not appear to recruit protein tyrosine phosphatase, but rather recruits the 5'inositol phosphatase SHIP (Scharenberg & Kinet, 1996, Ono *et al.*, 1996, Nadler *et al.*, 1997). The generation of mice deficient in FcγRIIb has highlighted the central role of this receptor in modulating immune responses and also in the prevention of autoimmunity (section 1.2.8.13) (Nakamura *et al.*, 2000, Clynes, Dumitru & Ravetch, 1998, Yuasa *et al.*, 1999).

1.2.8.4. FcγRIIb negatively regulates B-cell receptor activation

The BCR comprises surface Ig (sIg), and ITAM bearing signalling chains (α and β chains). The binding of antigen to the BCR via sIg is a critical step in B-cell activation, ultimately resulting in antigen specific B-cell proliferation and antibody production. Physiologically, the BCR and FcγRIIb are co-aggregated when the B-cell encounters immune complexes of its cognate antigen. Here, the BCR and FcγRIIb simultaneously bind these immune complexes, through the antigenic determinants and Fc region of IgG respectively. This 'co-ligation' results in a downregulation of the B-cell response, thereby providing a negative feedback loop to regulate antibody secretion (Scharenberg & Kinet, 1996).

1.2.8.5. FcγRIIb inhibits FcεRI induced degranulation of murine mast cells

A substantial body of work has implicated FcγRIIb as a negative regulator of murine mast cell degranulation induced by IgE. Aggregation of FcεRI on mast cells by IgE:allergen complexes leads to the rapid release of inflammatory mediators, such as histamine (degranulation). This response is mediated by the ITAM bearing γ-chain and β-chain subunits of FcεRI. Co-aggregation of FcγRIIb with FcεRI attenuates these responses (Ono *et al.*, 1996, Fong *et al.*, 1996). SHIP appears to be the inhibitory enzyme involved in this process as mast cells derived from SHIP knockout mice are hyper-responsive to IgE complexes (Huber *et al.*, 1998). Interestingly, FcγRIIb has been reported to show low affinity binding for IgE and this provides a physiological mechanism for the recruitment of FcγRIIb to IgE:antigen complexes (Choi *et al.*, 1996). Mast cell degranulation induced

by aggregation of the low affinity IgG receptor FcγRIII is also attenuated by FcγRIIb coaggregation.

1.2.8.6. The mechanism of FcγRIIb mediated inhibition

The molecular mechanisms governing FcγRIIb mediated inhibition have been largely defined using the B-cell model. Following co-aggregation of the BCR with FcγRIIb, the ITIM motif within the tail of FcγRIIb undergoes tyrosine phosphorylation. This phosphorylation creates a docking site for the SH2 domains of three phosphatases, SHP-1, SHP-2 and the inositol phosphatase, SHIP (SH2 containing Inositol Phosphatase) (Ono *et al.*, 1996, Dambrosio *et al.*, 1995, Dambrosio, Fong & Cambier, 1996a). In contrast to the KIR ITIM bearing receptors, use of the motheaten (me/me) mice (section 1.2.8.2) demonstrated that SHP-1 was not involved in mediating the actions of FcγRIIb (Ono *et al.*, 1996, Gupta *et al.*, 1997, Nadler *et al.*, 1997). A role for SHIP was proposed as phosphorylated FcγRIIb in both B-cells and mast cells has been reported to recruit SHIP. In addition, studies with dominant negative mutants of SHIP and SHIP knockout mice (section 1.2.8.14) has confirmed the critical role of this phosphatase in mediating the FcγRIIb inhibitory signal (Gupta *et al.*, 1997, Liu *et al.*, 1998).

1.2.8.7. SHIP is an inositol polyphsphate 5'phosphatase

SHIP was initially cloned as a 145kDa phosphoprotein found to associate with the adapter protein, Shc, in response to multiple cytokines (Ware *et al.*, 1996, Damen *et al.*, 1996). This protein consists of an N-terminal SH2 domain, a highly conserved inositol polyphosphate 5' phosphatase motif, two consensus sequence targets for PTB (phospho-tyrosine binding) domains and a proline rich region. SHIP selectively hydrolyses the 5' phosphate from phosphatidyl inositol 3,4,5-trisphosphate (PIP_3) and inositol 1,3,4,5-tetrakisphosphate ($InsP_4$). Multiple forms of SHIP have been reported which are thought to be generated by post-translational C-terminal truncation (Damen *et al.*, 1998), however, the physiological relevance of these truncated forms is not yet known.

1.2.8.8. SHIP uncouples receptors from PI 3-kinase / PIP_3 dependent signalling pathways

The major *in vivo* substrate of SHIP is believed to be PIP_3, which is a major product of PI 3-kinase activation (section 1.2.3). PIP_3 has emerged as a key translocation signal for multiple intracellular signalling components (section 1.2.3.2). Dephosphorylation of PIP_3 by SHIP generates $PI(3,4)P_2$. Through this process, the PIP_3 signal is attenuated and, therefore, SHIP is able to uncouple receptors (such as the BCR) from a number of key signalling pathways. Interestingly, following co-aggregation of the BCR with FcγRIIb, SHIP has been reported to directly associate with the p85 subunit of PI 3-kinase (Gupta *et al.*, 1999). The role of this interaction is not entirely clear, though it could imply that SHIP directly inhibits PI 3-kinase activity as well as hydrolysing its products.

1.2.8.9. SHIP inhibits calcium signalling by blocking Tec kinase dependent PLCγ activation

Elevation of cytosolic calcium through both mobilisation of intracellular stores and influx from the extracellular space, is a critical component of both antigen and Fc receptor signalling. As previously described (section 1.2.3.5), mobilisation of calcium is dependent on Tec kinase activation of PLCγ to generate $InsP_3$. Further, Tec kinase activation of PLCγ is dependent on the generation of PIP_3 by PI 3-kinase. Thus, by recruiting SHIP to the plasma membrane, FcγRIIb is able to direct the hydrolysis of PIP_3 and inhibit Tec kinase translocation and subsequent PLCγ activation and calcium influx (Bolland *et al.*, 1998, Scharenberg *et al.*, 1998).

1.2.8.10. FcγRIIb inhibits BCR induced Ras activation

FcγRIIb co-aggregation also inhibits BCR induced Ras activation. The mechanism governing this inhibition is still controversial. There are currently three proposed models. Firstly, the GTPase exchange factors (GEFs), Sos and Vav, contain PH domains capable of interacting with PIP_3 (section 1.2.3.2). By hydrolysing PIP_3, SHIP may inhibit Ras activation by preventing translocation of exchange factors to the plasma membrane. Secondly, a well-documented interaction has been demonstrated between SHIP and the

adapter proteins, Shc and Grb-2. These adapter proteins have been implicated in BCR mediated Ras activation where they serve to recruit Sos to the plasma membrane through the formation of Shc:Grb-2:Sos complexes (see section 1.2.5.2). It has therefore been proposed that SHIP may thus indirectly inhibit Ras activation by competing with Sos for binding to Shc and/or Grb-2 (Tridandapani *et al.*, 1998, Tridandapani *et al.*, 1997). Thirdly, a role for the Ras GTPase activating protein, RasGAP, in mediating FcγRIIb inhibition has been demonstrated (Tamir *et al.*, 2000). Here, RasGAP is recruited by the adapter protein, p62 DOK, in response to FcγRIIb co-aggregation with the BCR. This interaction is dependent on SHIP recruitment to the ITIM of FcγRIIb. By activating RasGAP, FcγRIIb could enhance the conversion of active Ras-GTP to inactive Ras-GDP.

1.2.8.11. The role of SHIP:Shc interactions

Multiple stimuli, including BCR aggregation, Fc receptor aggregation and cytokine activation, induce the association of SHIP with the adapter protein, Shc (Damen *et al.*, 1996, Cameron & Allen, 1999). This SHIP-Shc interaction is thought to involve the binding of the PTB domain of Shc to phosphorylated SHIP (Lamkin *et al.*, 1997). Defining the role of this interaction, however, has proved difficult. The recent generation of a SHIP deficient B-cell line has led to the surprising finding that Shc phosphorylation is dependent on the expression of SHIP (Ingham *et al.*, 1999). This result suggests that SHIP is upstream of Shc phosphorylation and, thus potentially, upstream of the Ras/MAP kinase pathway. As SHIP-Shc interactions can be induced in the absence of FcγRIIb co-aggregation, it seems likely that this novel role for SHIP is independent of its inhibitory actions.

1.2.8.12. FcγRIIb inhibits BCR induced Akt/PKB activation

In B-cells, FcγRIIb co-aggregation partially inhibits Akt activation (Jacob *et al.*, 1999, Aman *et al.*, 1998). As Akt activation depends on PIP_3, this inhibition is likely to be a result of SHIP mediated PIP_3 hydrolysis. Interestingly, $PI(3,4)P_2$, the product of PIP_3 hydrolysis by SHIP has been proposed to positively regulate Akt activity. Thus, SHIP

induced PIP_3 hydrolysis might act by inhibiting the kinases upstream of Akt activation (i.e. PDK1 and PDK2 - see section 1.2.3.3) rather than directly on Akt itself.

1.2.8.13. Targeted disruption of FcγRIIb

The importance of FcγRIIb in modulating B-cell proliferation and antibody secretion *in vivo* has been demonstrated by the generation of FcγRIIb deficient mice (Ono *et al.*, 1996). Mice which lack FcγRIIb show greatly enhanced antibody responses when challenged with antigen (Ravetch & Clynes, 1998). An additional role for FcγRIIb in the modulation of macrophage activation by Fcγ receptors in murine systems has been revealed using the FcγRIIb knockout mice (Clynes *et al.*, 1999). Interestingly, these studies have demonstrated a role for FcγRIIb in the control and prevention of auto-immune and inflammatory diseases (Nakamura *et al.*, 2000, Yuasa *et al.*, 1999, Clynes *et al.*, 1999). Thus, mice which lack FcγRIIb show greater susceptibility to, and severity of, disease in a number of auto-immune and inflammatory models. Clearly these studies reveal as central role for FcγRIIb in setting the thresholds for antigen and immune complex mediated activation.

1.2.8.14. Targeted disruption of SHIP

The role of SHIP as a crucial negative regulator of the immune system has been confirmed by generation of SHIP knockout mice. As expected, FcγRIIb mediated inhibition of B-cell and mast cell activation is disrupted in these animals, confirming the role of SHIP as the primary effector of FcγRIIb in these cells (Liu *et al.*, 1998, Huber *et al.*, 1998). Interestingly, BCR responses are enhanced in SHIP deficient B-cells even in the absence of FcγRIIb coaggregation. Further, these studies have revealed a role for SHIP in the downregulation of cytokine and chemokine signalling (Helgason *et al.*, 2000, Helgason *et al.*, 1998, Kim *et al.*, 1999). Important roles for SHIP in both T-cell and B-cell development have also been reported.

1.3. Monocytes and Macrophages

Macrophages participate in the production, activation and regulation of all immune effector cells. These cells belong to the mononuclear phagocyte lineage, being derived from blood monocytes which terminally differentiate to give rise to tissue macrophages. Probably the most significant role of these cells is to engulf and destroy foreign and aberrant material, including infectious agents, immune complexes, virus infected cells and cancer cells. These cells also play a key role in tissue homeostasis. Due to the differing roles of macrophages throughout the body, macrophages are highly hetergeneous.

1.3.1 The role of monocytes and macrophages in innate and adaptive immunity

Monocytes and macrophages have central roles in innate and adaptive immunity (Gordon, 1999). Receptors on the surface of these cells directly bind to pathogens and result in internalisation and disposal. These cells also express receptors for the complement system and C-reactive protein (CRP) which mediate internalisation of complement and CRP opsonised pathogens. These innate functions of the immune system serve as a first line of defence against pathogens. In the adaptive response, production of antigen specific antibodies by B-cells results in either the formation of antibody:antigen complexes, known as immune complexes, or the opsonisation of pathogens through antibody binding to surface antigens. The antibodies are then recognised by Fc receptors and the resultant aggregation of these receptors by immune complex or opsonised particle leads to their internalisation through the process of endocytosis for immune complexes or phagocytosis for opsonised particles. The focus of this section will deal primarily with the response of monocytes and macrophages to challenge with immune complexes.

1.3.2. Monocytes and macrophages orchestrate the inflammatory response

Inflammation is the physiological process which occurs in response to damage induced by multiple agents, including pathogens, foreign bodies, complement opsonised particles, and

immune complexes, as well as by physical injury (Rosenberg & Gallin, 1999). In the inflammatory response, soluble mediators and cellular components act in concert to eliminate the cause of damage. While this response is key to host defence, it is also clear that inflammation can result in tissue destruction associated with some disease processes. In inflammation, soluble inflammatory mediators are released which recruit and activate the cellular components of inflammation, including neutrophils, monocytes and lymphocytes. Many of these proinflammatory mediators are synthesised and released by macrophages and monocytes. These include the cytokines TNF-α, IL-1 (α and β), IL-6 and IL-8 together with other inflammatory mediators such as prostaglandins and eicosanoids. Further, in the resolution of inflammation, macrophages are responsible for clearing recruited leukocytes, such as neutrophils and for the release of anti-inflammatory mediators such as transforming growth factor β (TGF-β) and IL-10. Thus, monocytes and macrophages play a key role in the initiation, coordination and resolution of the inflammatory response.

Other cells also play key roles in the inflammatory response (Rosenberg & Gallin, 1999). Release of IFN-γ by NK cells and T-cells is important in the priming and activation of monocytes and macrophages. Neutrophils are phagocytic cells, which are rapidly recruited in response to inflammatory stimuli. These cells play a key role in the clearance of foreign materials and cellular debris. They, therefore, express high levels of phagocytic receptors, including Fc receptors and complement receptors. Eosinophils and mast cells play a central role in inflammation associated with allergy. Central to this response are IgE and its receptors (e.g. FcεRI and CD23) expressed on these cells.

1.3.3. Monocytes and macrophages contribute to lymphocyte regulation

Alongside their central roles in immune complex clearance and inflammation, monocytes and macrophages, also interact and coordinate lymphocyte responses. Monocytes and macrophages are also a major source of the immune regulatory cytokines IL-10 and IL-12 which regulate the type of lymphocyte response. In addition, macrophages are able to present internalised antigen to T-cells via MHC class II. This receptor is the most effective method of stimulating T-cell activation (Gordon, 1999).

1.3.4. Monocytes and Macrophages are highly heterogeneous

Mononuclear cells such as monocytes and macrophages are highly heterogeneous, attributable to differences in their maturation status and also tissue specific variation. This heterogeneity makes *in vitro* study of these primary cells very difficult. Several murine models for studying primary mononuclear cells are widely used for the study of these cells. Here, the use of inbred mouse strains and tissue specific cell populations allows heterogeneity to be minimised. Further, the availability of transgenic technology in mouse systems has proved invaluable. A number of murine macrophage cell lines (i.e. J774) have also proved useful as they are easy to culture and relatively homogenous. Primary human mononuclear cells provide more difficulty due to genetic variation and disease status differences between donors. To overcome this difficulty, a number of human monocyte-like cell lines have been established, including U937, HL60 and THP-1 (Harris & Ralph, 1985, Auwerx, 1991). These cells are widely used as models for studying human mononuclear cells, their functional properties and the changes they undergo during maturation.

1.4. The use of the U937 cell line in the study of Fcγ receptor biology

The U937 cell line was established from the pleural exudate of a patient with diffuse histocytic lymphoma (Sundstrom & Nilsson, 1976). U937 cells share many characteristics of immature monocytes. These cells constitutively express FcγRI and FcγRIIa, but not FcγRIII. Many of the effector functions mediated by these cells require stimulation of U937 cells with agents such as IFNγ, dibutyryl cyclicAMP (dbcAMP), phorbol esthers and vitamin D (Harris & Ralph, 1985). Thus, treatment of U937 cells with IFN-γ induces the rapid upregulation of the high affinity receptor FcγRI. Further, IFNγ increases expression of MHC class II, and enhances Fcγ receptor mediated phagocytosis and reactive oxygen metabolism. IFN-γ primed U937 cells, therefore, provide a model for studying activated monocytes. Alternatively, U937 cells can be induced to terminally differentiate to become more macrophage-like cells with agents such as dbcAMP and PMA (Sheth *et al.*, 1988). Treatment of cells with dbcAMP induces

upregulation of the low affinity receptor FcγRIIa with a concomitant downregulation of FcγRI.

In our laboratory, we use the U937 cell line to study signalling events activated by immune complexes. As U937 cells undergo controlled differentiation when treated with agents such as IFN-γ and dbcAMP, we are able to investigate the molecular mechanisms which link immune complexes to the activation of specific signalling pathways and cellular responses.

1.4.1. Signalling pathways activated by FcγRI in U937 cells

The human high affinity receptor for IgG, FcγRI has no known signalling motif within its cytoplasmic tail (Allen & Seed, 1989). In myeloid cells, including U937 cells, FcγRI non-covalently associates with γ-chain homodimers which bear cytoplasmic ITAM motifs for the activation of soluble tyrosine kinases (see section 1.2.2).

1.4.2. Differentiation of U937 cells alters the signalling pathway activated by FcγRI to mobilize calcium

Previous work in our laboratory has demonstrated that differentiation of cells with either IFN-γ or dbcAMP, changes the nature of the calcium responses activated by FcγRI (Davis, Sage & Allen, 1994, Davis *et al.*, 1995a, Melendez *et al.*, 1998a). Thus, in IFN-γ primed U937 cells, aggregation of FcγRI induces a single calcium spike resulting solely from the release of calcium from intracellular stores. In these cells, FcγRI aggregation results in virtually no calcium influx. Conversely, in U937 cells differentiated to more macrophage like cells with dbcAMP, FcγRI aggregation results in both store release of calcium and calcium influx (Davis *et al.*, 1995a).

Melendez *et al* have demonstrated that the switch in the nature of calcium transients results from a fundamental switch in the signalling pathway activated by FcγRI under the different conditions of differentiation (Melendez *et al.*, 1998a). Thus, in dbcAMP treated cells, FcγRI couples to the classical calcium mobilisation pathway described in section

1.2.4. Here, receptor aggregation results in PLCγ activation, production of $InsP_3$ and release of calcium from internal stores (Melendez, Harnett & Allen, 1999b). This calcium store release results in the opening of the plasma membrane calcium channel, I_{CRAC}, to generate calcium oscillations. Conversely, in IFN-γ primed U937 cells, FcγRI couples to a novel pathway, involving the sequential activation of PI 3-kinase, PC-PLD and sphingosine kinase. The resultant cytosolic calcium response results from store release, apparently independent of PLCγ activation and $InsP_3$ production. Here, no calcium influx is observed in response to FcγRI aggregation.

1.4.3. FcγRI activates a novel PLD/Sphingosine Kinase pathway to mobilize calcium in IFNγ primed U937 cells.

In IFN-γ primed U937 cells, FcγRI aggregation results in release of calcium from intracellular stores. However, this release of calcium stores is not accompanied by activation of PLCγ to generate $InsP_3$. Rather, FcγRI couples to the activation of PC-PLD and downstream activation of sphingosine kinase (Melendez *et al.*, 1998b). The cloning of the SCaMPER receptor provides a pathway whereby sphingoid derivatives could effect calcium release from the endoplasmic reticulum (Mao *et al.*, 1996). This novel, $InsP_3$ independent pathway to calcium release remains the subject of some controversy.

1.4.4. The differentiation dependent switch in signalling pathway activated by FcγRI appears to depend on a switch in the accessory molecule recruited by FcγRI.

As discussed above, FcγRI couples to PLCγ in dbcAMP differentiated U937 cells to mobilise calcium whereas in IFN-γ primed cells FcγRI couples to PC-PLD to mobilize calcium. Interestingly, the use of mAbs to activate the low affinity receptor, FcγRIIa, demonstrated that this receptor is coupled to the activation of the PLCγ pathway, but not PC-PLD, regardless of the differentiation state of the cell (Melendez *et al.*, 1998a). Thus, in both IFN-γ primed and dbcAMP differentiated cells, FcγRIIa activates PLCγ and elevates $InsP_3$. Furthermore, activation of PLCγ by FcγRIIa in either cell type was associated with both calcium store release and calcium influx. Taken together, this raised

the hypothesis that FcγRI might recruit FcγRIIa in dbcAMP treated cells to activate PLCγ. Alternatively, FcγRI might couple to PC-PLD through the γ-chain in IFN-γ primed cells. To test this theory antisense oligonucleotides against FcγRIIa and γ-chain were employed to specifically reduce levels of these proteins. Consistent with the above theory, anti-sense oligonucleotides to FcγRIIa reduced levels of PLCγ activated by FcγRI in dbcAMP differentiated cells, but had no effect on PLD activation in IFN-γ primed cells. Conversely, anti-sense oligonucleotides to the γ-chain decreased PLD activation in IFN-γ primed cells but had no effect on FcγRI mediated PLCγ activation in dbcAMP differentiated cells. The data from these experiments implied that in dbcAMP differentiated cells, FcγRI is coupled to the activation of PLCγ through recruitment of FcγRIIa, whereas in IFN-γ primed cells, FcγRI is coupled to the activation of PC-PLD through recruitment of the γ-chain (Melendez *et al.*, 1998a) (see Chapter 6).

1.4.5. Differentiation dependent switch in PKC isoform activation by FcγRI in U937 cells

As discussed in section 1.2.4.4, activation of specific PKC isoforms is dependent on second messengers such as calcium and DAG. As FcγRI induces differing calcium transients in IFN-γ and dbcAMP differentiated U937 cells, it seemed likely that different PKC isoforms would be activated by FcγRI. Consistent with the short calcium signal in IFN-γ primed cells, FcγRI is coupled to calcium independent PKC isoforms (δ, ε, ζ). In dbcAMP differentiated cells, where FcγRI aggregation induces prolonged calcium oscillations, the receptor is coupled to the activation of calcium dependent PKC isoforms (α, β, γ). Thus, differentiation of U937 cells results not only in a switch in the nature of the activated phospholipase pathway activated, but also in a switch in the activation of PKC isoforms (Melendez, Harnett & Allen, 1999a).

1.5. Objectives of this study

The signalling pathway activated by FcγRI in U937 cells depends strictly on the differentiation state of the cell. Thus, in IFN-γ primed cells, FcγRI is coupled to the activation of PC-PLD and sphingosine kinase resulting in the release of calcium from intracellular stores but no calcium influx. In cells induced to differentiate to a more macrophage-like phenotype with dbcAMP, FcγRI is coupled to PLCγ, calcium release from stores and calcium influx.

The aim of this project was to examine the mechanisms governing this differentiation dependent switch in signalling pathways activated by FcγRI in U937 cells. Two parallel approaches were taken:

1) To examine the potential of FcγRI to activate inhibitory pathways in either IFN-γ or dbcAMP differentiated cells. In IFN-γ primed U937 cells, FcγRI couples to PLD activation with no activation of PLCγ. However, the PLCγ pathway is intact in these cells as aggregation of FcγRIIa in identical cells results in the activation of PLCγ. This raises the possibility that FcγRI might activate inhibitory signals to block the activation of PLCγ. As the 5' inositol phosphatase, SHIP, has been shown to block PLCγ activation downstream of a number of immune receptors, the possible involvement of this pathway was of particular interest.

2) To elucidate the molecular basis for the switch in FcγRI coupling to different phospholipase pathways Activation of PLCγ or PC-PLD by FcγRI appears to depend on the recruitment of different accessory molecules to transduce signals. Thus, in IFNγ treated cells, FcγRI appears to couple to PC-PLD activation via the γ-chain. Conversely, in dbcAMP cells FcγRI appears to couple to PLCγ via FcγRIIa. This raises the possibility that the switch in signalling pathways results from fundamental differences in the signalling motifs of the γ-chain and FcγRIIa. Here, the coupling of FcγRI to either γ-chain or FcγRIIa was addressed. Further, the potential involvement of specific molecular components implicated in the activation of distinct phospholipase pathways were examined under different signalling conditions.

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Biochemicals

Unless otherwise stated, all chemicals were purchased from Sigma.

2.1.2. Immunochemicals

The commercial antibodies used in this study are listed in table 2.1.

2.2. Cell biology techniques

2.2.1. Cell culture

2.2.1.1. U937 cell line

U937 cells (Sundstrom & Nilsson, 1976) were routinely cultured in RPMI 1640 medium (Imperial Laboratories) supplemented with foetal calf serum (10% v/v), glutamine (2 mM), penicillin (10 U/ml) and streptomycin (10 μg/ml) at 37°C, 6.8% carbon dioxide. Cells were maintained at a density of between 5 x 10^5 and 2 x 10^6 cells/ml, as measured using a Neubauer haemocytometer, and were routinely sub-passaged twice weekly. To sub-passage, cells were harvested by centrifugation for 5 min at 400 g and resuspended in fresh complete medium. Typically cells were maintained in 75 cm^2 tissue culture flasks in a volume of 10 mls.

2.2.1.2. U937:Δp85 cell line

The U937:Δp85 cell line was provided as a kind gift by L. Stephens (Babraham Institute, Cambridge, UK). Cells were maintained as for normal U937 cells with the addition of 0.1 mg/ml Hygromycin B and 0.6 mg/ml G418 (both from Calbiochem). Δp85 expression was induced with 15 mM isopropyl β-D thiogalactoside (IPTG), 5 nM phorbol 12-myristate 13-acetate (PMA), and 100 μM zinc chloride for 10 hours. Control cells were treated with the same levels of PMA and zinc chloride in the absence of IPTG.

2.2.1.3. Differentiation of U937 cells with IFN-γ and dbcAMP

U937 cells were primed in fresh medium with IFN-γ (200 ng/ml) where indicated for 24 hours prior to experiments. Alternatively, cells were induced to differentiate by culturing in 1 mM dibutyryl cyclicAMP (dbcAMP) for 48 hours prior to experiments.

2.2.2. Flow cytometry

2.2.2.1. Analysis of surface receptor expression with monoclonal antibodies

Typically $1x10^6$ cells were washed with ice cold PBS (0.07 M Sodium Chloride, 75 mM disodium hydrogen orthophosphate, 5 mM sodium dihydrogen orthophosphate) and resuspended in 100μl PBS with 1μg of relevant monoclonal antibody (mAb). Where appropriate, 3 μM monomeric human IgG was added to block non-specific interactions of monoclonal antibodies with Fc receptors. Following 45 mins incubation on ice cells, were washed twice and incubated for a further 45 mins with a fluoroscein isothiocyanate (FITC) conjugated goat anti-mouse IgG secondary antibody (1:100 dilution). Irrelevant isotype control antibodies were used to control for non-specific labeling. Cells were analysed using a Becton Dickinson FACScan.

2.2.2.2. IgG:FITC binding and receptor blocking

For IgG binding, 10^6 cells were incubated with 1 μg of FITC conjugated human IgG (IgG:FITC, Sigma) in 100 μl for 30 min on ice. In chapter 6, specific mAbs are used to

block the ligand binding pockets of specific receptors to assess the effect on IgG binding. To block the ligand binding pocket of FcγRI or FcγRIIa, cells were pre-incubated with mAb 10.1 (20 μg/ml) or mAb IV3 (20 μg/ml) respectively for 20 min prior to the addition of IgG:FITC. Cells with no IgG:FITC were used to control for autofluorescence. Cells were analysed using a Becton Dickinson FACScan.

2.3. Biochemical assays and methods

2.3.1. Fc receptor aggregation

2.3.1.1. FcγRI aggregation with human IgG

Cells were harvested by centrifugation (5 min, 400 g), washed and resuspended in ice cold RPMI / 25 mM HEPES, and incubated with 1μM monomeric IgG (Serotec) for 45 mins at 4°C to occupy surface FcγRI. Cells were then washed twice with RPMI to remove unbound ligand. Following this cells were resuspended in RPMI, 25 mM HEPES, 1% FCS and a 1:50 dilution of goat anti-human IgG (Serotec) was added to crosslink FcγRI bound IgG. Cells were then warmed to 37°C for the times specified. To block the ligand binding pocket of FcγRIIa (see chapter 6), cells were pre-incubated mAb IV3 (20 μg/ml) respectively for 20 min prior to the addition of goat anti-human IgG.

2.3.1.2. Fc receptor aggregation with monoclonal antibodies

To aggregate specific receptors, cells were washed and incubated with specific mAbs for 45 min at 4°C. Cell were washed to remove unbound mAbs and the receptors were cross-linked by the addition of goat anti-mouse IgG (1:50 dilution; Sigma). Cells were then warmed to 37°C for the times specified.

2.3.2. Determination of protein concentration

Protein concentrations were routinely determined for all cell lysates and membrane preparations. Protein concentrations were estimated using the Bio-Rad protein assay system which is based on the Bradford Coomassie brilliant blue dye binding system. A range of bovine serum albumin (BSA) standards was used to construct a standard curve and protein samples were suitably diluted to fall within the linear range of this curve. Protein concentrations of all samples were routinely measured in duplicate.

2.3.3. Cell lysate preparation

For general lysate preparations, cells in suspension were lysed by the addition of ice-cold lysis buffer (1% (v/v) Triton X-100, 50 mM Tris-HCl, pH 7.5, 0.25% (w/v) sodium-deoxycholate , 150 mM NaCl, 1 mM EDTA, 1 mM vanadate and 1 mM NaF) containing protease inhibitors (1 mM phenylmethylsulfonyl flouride and 1 μg/ml each chymostatin, leupeptin, antipain and pepstatin). Alternatively, where the interactions between membrane associated proteins needed to be preserved (such as the interaction between FcγRI and γ-chain) a digitonin based lysis buffer was used (1% (v/v) Digitonin, 0.12% (v/v) Triton X-100, 20 mM Tris-HCl, pH 7.5), 150 mM NaCl) also containing the above listed protease inhibitors. Following 30 min tumbling at 4°C lysates were clarified at 15 000 g for 15 mins to remove insoluble cell debris. Lysates were either used immediately for immune precipitations or stored at -70 °C prior to electrophoresis.

2.3.4. Immune precipitation

A large number of immune precipitations were carried out during the course of my PhD studies and protocols varied considerably. Outlined below are typical protocols only. For the specific method used for any given immune precipitation, please refer to the relevant results chapters.

2.3.4.1. Protein G agarose protocol

Typically 500 μg of cell lysate for each sample was precleared with 20 μg protein G agarose (Santa Cruz Biotechnology) for 1 hour at 4°C to reduce non-specific binding. For

standard immune precipitations, 2 µg of specific antibody pre-conjugated to 10 µg protein G agarose was added to each sample and incubated by tumbling overnight at 4°C. Precipitates were collected by centrifugation (400 g, 5 min) and washed four times with 1 ml of lysis buffer (see section 2.3.3). Immune precipitates were subjected to Western blot analysis (sections 2.3.5 and 2.3.6) or inositol phosphatase assays (section 2.4.2).

2.3.4.2. Direct immobilisation of antibodies to agarose

To overcome high levels of background binding experienced using protein G agarose, antibodies for many immune precipitations were directly conjugated to agarose beads using the Pierce Amino Link Plus Immobilisation system. The agarose provided with this system has been activated to form aldehyde functional groups which react with primary amines present on proteins. Reduction of the resulting Schiff bases with sodium cyanoborohydride allows stable covalent coupling of antibodies to the agarose beads. In this kit, 4% beaded agarose is supplied as a 50% slurry in columns. For the purposes of immobilising antibodies for immune precipitations, the agarose was removed from the columns and aliquoted into 1.5 ml eppendorfs. All immobilisations were carried out at pH 7.2 in PBS according to the manufacturers protocol. As the agarose was no longer in columns, all washing steps were carried out by repeated dilution and centrifugation. Following immobilisation, agarose conjugated antibodies were stored at 4°C in PBS with 0.05% (w/v) sodium azide as a preservative. In many cases secondary precipitating antibodies were immobilised to reduce cost and the number of immobilisations required. For example, large batches of polyclonal anti-mouse IgG were immobilised to allow the precipitation of any primary mouse monoclonal antibody.

Typically 500 µg of cell lysate was precipitated with 2 µg of specific antibody at 4°C overnight. In cases where immobilized secondary antibodies were used to precipitate specific antibodies, the secondary antibodies were added in excess. Immune precipitates were washed four times with lysis buffer prior to analysis.

2.3.5. Sodium dodecyl sulphate- polyacrylamide electrophoresis (SDS-PAGE)

Through the course of my PhD studies a number of different gel systems were used in analysis of protein by SDS-PAGE. Below are outlined the two systems used for general procedures.

2.3.5.1. Laemmli protocol

SDS-PAGE was carried out according to the procedures described by (Laemmli, 1970). All acrylamide used here had an acrylamide to bis-acrylamide ratio of 29:1 (w/w). Resolving gels contained 375 mM Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.06 % (w/v) ammonium persulphate and 0.08% (v/v) N,N,N',N'-tertramethylethylenediamine (TEMED). Acrylamide concentrations were varied from 6% to 12.5% (w/v) according to the required separation range. All resolving gels were overlaid with a 5% (w/v) acrylamide stacking gel containing 125 mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.06 % (w/v) ammonium persulphate and 0.1% (v/v) TEMED. Protein samples were denatured by boiling for 5 minutes in 1x SDS-PAGE sample buffer (50 mM Tris-HCl, pH 7.5, 1% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10 mM EDTA, 10% (v/v) glycerol and 0.01% (w/v) bromophenol blue). Proteins were electrophoresed in an Hoeffer SE 600 series vertical slab gel unit at a constant voltage (50 - 200V) until the bromophenol blue tracking dye reached the bottom of the gel. For Western blotting, proteins were transferred to nitrocellulose as described below (section 2.3.6.).

2.3.5.2. Novex precast gel protocol

The novex NuPAGE precast gel system (Invitrogen-Novex) offers a number of advantages, over standard SDS-PAGE systems in terms of speed, resolution and transfer efficiency. The NuPAGE system works with bis-tris buffered (pH 6.4) polyacrylamide gels, available at 3 different acrylamide concentrations (10% (w/v), 12% and 4-12% gradient gels) allowing different separation ranges to be selected. Further, by varying the running buffer used, the separation range can be varied further. Samples are denatured in NuPAGE LDS (Lithium Dodecyl Sulphate) sample buffer by heating to 70°C for 10 min.

The decreased denaturing temperature prevents sample degradation and precipitation prior to electrophoresis. For reduced proteins, 50 mM dithiothreitol was added prior to sample denaturing. For a detailed protocol, including gel separation ranges, please refer to the manufacturers instructions.

2.3.6. Western blot analysis

For Western blot analysis, proteins were transferred to nitrocellulose (Schleicher and Schuel GmbH) using a Hoeffer TS series, Transphor Electrophoresis Unit. Transfer was carried out in 190 mM glycine, 25 mM Tris base, containning 20% (v/v) methanol at 300 mA for 4 hours. Alternatively, where Novex precast gels were used, transfer was carried out according to the manufacturers instructions using the Novex XCell II blotting apparatus. Transfer efficiency was ascertained by stainning with Ponceau S solution for 5 minutes followed by washing with double distilled H_2O. Ponceau S was removed by washing with TBS (20 mM Tris.HCl, pH 7.6, 136 mM NaCl) containing 0.1% (v/v)Tween 20 (TBST). Nitrocellulose blots were blocked in TBST with 4% (w/v) non-fat powdered milk (Marvel) for a minimum of 1 hour at room temperature. Typically, blots were incubated with primary antibodies at a final concentration of 0.1-1 μg/ml (Table 2.1) in TBS overnight at 4°C, Blots were then washed three times for 5 minutes with TBST. Blots were incubated with an appropriate secondary antibody (1:1000 to 1:5000 dilution - see table 2.1) for 1-4 hours in TBST at room temperature. Blots were washed extensively with large volumes of TBST (typically 5x5 min washes) followed by a final wash in TBS prior to developing. Blots were developed using the Amersham enhanced chemiluminescence (ECL) system. To strip blots for reprobing, blots were washed in acid strip buffer (100 mM glycine, 150 mM NaCl, pH 2.6) for 45 minutes.

2.3.6.1. Detection of phosphotyrosine containing proteins by Western blot

Proteins phosphorylated on tyrosine residues can be detected by Western blot using the monoclonal antibody, 4G10 (Upstate Biotechnology). This antibody gives a very poor signal and high background using the standard blotting protocol outlined above (section 2.3.6) so the following modified protocol was developed. Following membrane blocking

with TBST 4% milk, blots were incubated with a 1:1000 dilution of 4G10 (Upstate Biotechnology) overnight at 4°C in TBS with 0.05 % (v/v) Tween 20 and 0.5 % (w/v) milk. Membranes were washed 3x5 minutes with TBST and incubated for 2 hours with donkey anti-mouse IgG: HRP (1:4000, Amersham) in TBST with 4% (w/v) milk. Membranes were then rinsed four times with large quantities of ddH_20, washed 5x5 min with TBST with vigorous agitation followed by four further rinses with ddH_20. Blots were developed as described above (section 2.3.6).

2.3.6.2. Detection of activated MAPK by Western blot (ERK, p38 and JNK)

To detect activated forms of the MAPKs, lysates were prepared as described in section 2.3.3. Typically, 10 μg of total cell lysate was electrophoresed and blotted for each sample using Novex precast gels as described in sections 2.3.5.2 and 2.3.6. Activated forms of ERK, p38 and JNK were detected by Western blot (section 2.3.6.) using rabbit polyclonal antibodies raised against peptides corresponding to the dual phosphorylated active sites of the different MAP kinases. All blots for activated MAPKs were stripped and reprobed with polyclonal antibodies which recognise all forms of the MAPK being assayed to ensure comparable loading of cell lysates between samples. For antibody specificities see table 2.1.

2.3.6.3. Detection of activated Akt/PKB by Western blot

Activated Akt/PKB is phosphorylated on a number of sites, including Serine 473 (Ser473). Activated PKB can be detected by Western blot as described for the MAPKs (section 2.3.6.2) using rabbit polyclonal antibodies which recognise PKB phosphorylated on Ser473 (phospho-PKB - see Table 2.1). All blots for activated phospho-PKB were stripped and reprobed with sheep polyclonal antibodies which recognise all forms of PKB to ensure comparable loading of cell lysates between samples.

2.3.7. Ras and Rac assays

The Ras and Rac1 small GTPases were assayed using Ras and Rac activation assay kits (Upstate Biotechnology). These kits comprise glutathione agarose bound GST fusion

proteins of the Ras binding domain (RBD) of Raf-1 (which binds Ras:GTP) or the p21 binding domain of PAK-1 (which binds Rac1:GTP). Briefly, cells were stimulated and then lysed with Mg^{2+} lysis buffer (MLB - Upstate Biotechnology). Typically, 500 µg cell lysate at a concentration of 1 mg/ml was used for each assay sample. Active Ras was precipitated with 5 µl Raf-1 RBD agarose for 30 minutes. Active Rac1 was precipitated with 5 µl PAK-1 PBD agarose for 60 minutes. Precipitates were washed with 3x500 µl MLB followed by SDS-PAGE (section 2.3.5.2) and Western blotting (section 2.3.6). Ras and Rac1 were detected with specific mAbs

2.3.8. Membrane preparation

Cells were washed twice in cold nuclear preparation buffer (10 mM Tris-HCl, pH 7.4, 2 mM magnesium chloride, 140 mM sodium chloride). Cells were then lysed by three liquid nitrogen freeze-thaw cycles in nuclear preparation buffer containing 2% (v/v) Tween 40, protease inhibitors (1 mM phenylmethylsulfonyl flouride and 1µg/ml each chymostatin, leupeptin, antipain and pepstatin) and phosphatase inhibitors (1mM vanadate and 1mM NaF). Cell debris and nuclei were removed by centrifugation (15 000 g, 5 min) and membranes were precipitated by ultracentrifugation (Beckman Instruments) of the supernatant at 100 000 g, 4°C for 1 hour (Bunce *et al.*, 1988, Divecha, Banfic & Irvine, 1991). Following ultracentrifugation, the supernatant represents the cytosolic fraction. Membrane pellets were resuspended in cold nuclear preparation buffer.

2.4. Biochemical assays

2.4.1. Measurement of of phosphatidyl choline Phospholipase D (PC-PLD) activity

PC-PLD activity was measured by the transphosphatidylation assay (Briscoe, Plevin & Wakelam, 1994). Briefly U937 cells were labeled (10^6 cells/ml) with [^{3}H] palmitic acid (5 µCi/ml) in RPMI 1640 containing FCS (10%) for 16 h. Following labeling, the cells were washed in ice-cold RPMI 1640 containing 10 mM HEPES and 0.1% (v/v) FCS (RHB medium) before being incubated (at a final concentration of 2 x 10^6 cells/ml) in RHB

medium containing 0.3% (v/v) butan-1-ol at 37 °C for 15 min. Specific Fc receptors were cross-linked for 30 min as described above (section 2.3.1.), and the cells were extracted by Bligh-Dyer phase separation. An aliquot of the lower organic phase was removed and dried down under vacuum and the samples were redissolved in 25 ml chloroform/methanol (19:1 v/v), containing 40 μg unlabeled phosphatidyl-butanol (Lipid Products, South Nutfield, Surrey, UK) as standard, and applied to pre-run, heat activated TLC plated (20 x 20 cm), Silica gel 150A groved plates (Whatman). The plates were developed in the organic phase of the solvent, ethyl acetate/1,2,4-trimethylpentane/acetic acid/water (11:5:2:10), for approximately 90 min, and the position of the phosphatidyl-butanol (PtdBut) product was detected using iodine vapour. [^{3}H] PtdBut-containing silica, as indicated by the standard, was then scraped into scintillation vials and counted. Results were calculated as a percentage of the total radioactivity incorporated in the lipids.

2.4.2. In vitro *inositol phosphatase assay*

Hydrolysis of [^{3}H]Ins(1,3,4,5)P_4 (NEN) by immune precipitates was measured in 25 μl containing 16 μM [^{3}H]Ins(1,3,4,5)P_4, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$. Reaction mixtures were incubated, with shaking, for 10 min at 37 °C, stopped with 500 μl ice cold water, and immediately applied to 0.5 ml dowex-formate columns. [^{3}H] Ins(1,3,4)P_3 product was eluted with 6 ml 0.7 M ammonium formate, 0.1 M formic acid and non-hydrolysed [^{3}H]Ins(1,3,4,5)P_4 was eluted with 6 ml 2.0 M ammonium formate, 0.1 M formic acid (Damen *et al.*, 1996, Mitchell, Connolly & Majerus, 1989). Both eluted fractions were counted in a Beckman Instuments scintilation counter.

2.5. Molecular Biology

2.5.1. RNA preparation

Total cellular RNA was isolated by acid phenol extraction. Briefly, cells were lysed by resuspending cell pellets in GTC lysis buffer (4 M guanidinium isothicyanate, 25 mM sodium citrate pH 7, 0.5 % (w/v) lauroylsarcosine, 100 mM β-mercaptoethanol). Samples were acidified with 0.2 M sodium acetate (pH 4), followed by the addition of an

equal volume of acidified phenol (pH 4). To ensure good phase separation chloroform : isoamylalcohol (24:1, 100 ul added/ml total volume) was added. Following vigorous vortexing cells were incubated on ice for 15 minutes and spun (1 500 g) at 4°C for 20 minutes to separate phases. The aqueous layer, which contains the RNA, was then removed to a fresh tube and the RNA precipitated with an equal volume of isopropanol followed by centrifugation (15 000 g, 20 min). RNA pellets were washed with 80% ethanol, resuspended in an appropriate volume of diethylpyrocarbonate (DEPC) treated water and stored at -70°C. RNA concentration was determined by measuring absorbance at 260 nm and purity was estimated by checking the 260 nm:280 nm absorbance ratio (RNA has a ratio of roughly 2:1).

2.5.2. Northern Blotting

For Northern blotting, equal quantities of total RNA (typically 20 μg) from each sample were denatured by heating to 60°C for 20 mins in the presence of 10% (v/v) formamide and 4% (w/v) formaldehyde. The RNA was then separated by electrophresis through a 1% agarose gel containing 0.02 M MOPS (pH 7), 1 mM EDTA and 0.6 (w/v) formaldehyde. 10 mM iodoacetamide was also added to the gel as an RNAse inhibitor. To visualise RNA the gel was stained with 0.5 μg/ml ethidium bromide for 30 mins. RNA was transferred to nitrocellulose (Amersham) overnight by capillary elution with 20xSSC (3 M NaCl, 0.3 M Sodium Citrate, pH 7) as transfer buffer. RNA was fixed to the nitrocellulose membrane by irradiating with ultraviolet light. The positions of 28S and 18S ribosomal RNAs were marked on the blots to allow size estimation of specific mRNA bands. Blots were typically hybridized overnight at 55°C with a ^{32}P labeled double stranded DNA probe in 0.5 M sodium phosphate (pH 7.2), 6.6% (w/v) SDS, 4 mM EDTA and 6.7 mg/ml polyA.

2.5.3 ^{32}P labeling of DNA

2.5.3.1. ^{32}P random primer labeling of double stranded DNA

To generate radiolabeled probes for hybridising to blots, double stranded DNA templates were labeled using the High Prime (Boehringer Mannheim) random primer labeling system. Specific template DNA was excised from the appropriate cDNA clone by restriction digestion (section 2.5.4.). Following electrophoresis, the correct DNA fragment was excised from the gel and purified using a Qiagen gel extraction kit. Alternatively, restriction digests were electrophoresed in low melting point agarose to allow labeling directly from the excised gel slice. Template DNA was denatured by heating to 100°C for 10 minutes prior to labeling. Approximately 10 ng DNA was labeled in 20 µl containing 4 µl Highprime reaction mix (contains buffers, Klenow fragment of DNA polymerase I, dATP, dGTP and dTTP), and 5µl [α-^{32}P] dCTP (1.85MBq at approx. 110 TBq/mmol -Amersham). Reactions were carried out at 37°C for 10 minutes. If low melting point agarose was used incubation time was increased to 1 hour. Labeled probe was separated from unincorporated label using Clontech size exclusion spin columns (Chroma Spin-30). Labeled probes were boiled for 10 minutes prior to hybridization to Northern blots (section 2.5.2.) or Southern blots (section 2.5.8.1.).

2.5.3.2. ^{32}P 5' end labeling of oligonucleotide probes with polynucleotide kinase

T4 polynucleotide kinase (T4-PNK) catalyses the transfer of the γ-phosphate from ATP to the 5'-hydroxyl terminus of DNA. 10 pmolel of specific oligonucleotide was mixed with 15 µl [γ-32P] dATP (5.55 MBq at approx. 110 TBq/mmol -Amersham), 5 µl 10x kinase buffer (Promega), 10 units (1 µl) T4 PNK (Promega) in a total of 50 µl. Following a 10 minute incubation at 37°C, reactions were terminated by the addition of 2 µl 0.5 M EDTA. Labeled probes were hybridised to PCR Southern blots (2.5.8.1.)

2.5.4. Restriction enzyme digestion of plasmid DNA

Typically 1 µg plasmid DNA was digested in 25 µl containing 2.5 µl of an appropriate reaction buffer and 1 µl of enzyme. If two restriction enzymes were used then 0.7 µl of each enzyme was used to keep glycerol concentrations to a minimum. Reactions were

incubated for 1 hour at 37°C. All restriction enzymes were purchased from Boehringer Mannheim and appropriate buffers were chosen using the supplied buffer compatibility chart. If larger quantities of DNA needed to be digested then reaction times and/or volumes were increased accordingly.

2.5.5. Agarose gel electrophoresis of DNA

Restriction digested DNA and PCR products were typically electrophoresed at 60 V through 1-2% (w/v) agarose in TAE buffer (40mM Tris-acetate, 1mM EDTA) containing 0.5 μg/ml ethidium bromide. Bands were visualised on an ultraviolet transilluminator and photographed with a Polaroid instant camera.

2.5.6. Transformation of Escherichia Coli *DH5-α and JM109 for plasmid preparations*

For general plasmid preparations, competent subcloning efficiency DH5-α (GibcoBRL) or JM109 (Promega) cells were used. Briefly, 50 μl competent cells were gently mixed with 1-10 ng DNA and incubated on ice for 30 minutes. Cells were then heat shocked at 42°C for 30 seconds followed by a 2 minute incubation on ice. Following the addition of 1 ml LB medium (Lennox L Broth - GibcoBRL), cells were allowed to recover for 1 hour at 37°C prior to plating an aliquot on to LB agar plates (GibcoBRL) containing 100 μg/ml ampicillin. Colonies were grown overnight at 37°C.

2.5.7. Purification of plasmid DNA

For each plasmid preparation a single colony was picked from a freshly streaked ampicillin selective plate and innoculated into a 5 ml LB culture containing 100 μg/ml ampicillin. For minipreparations of plasmid DNA, 5 ml cultures were grown up overnight at 37°C prior to plasmid purification as outlined below (section 2.5.7.1.). Alternatively, when larger quantities of DNA were required, 5ml 'starter' cultures were incubated at 37°C for 8 hours prior innoculation into larger volumes. When using constructs in the CDM vector, 500 ml cultures were typically grown overnight and plasmid DNA purified by the caesium chloride method outlined below (section 2.5.7.2.).

For all other plasmids, 100 ml cultures were grown and plasmid DNA purified using Qiagen maxi-prep column purification according to the manufacturers protocol. For procedures where endotoxin contamination was considered a problem, such as transfection of U937 cells, EndoFree plasmid kits were used.

2.5.7.1. Minipreparations

For purification of small quantities of plasmids (1-5 μg) ,5 ml cultures were grown (section 2.5.7). Briefly, 1.5 mls of the cell culture was precipitated at 12 000 g for 30 seconds. The cell pellet was resuspended in 100 μl GTE buffer (50 mM glucose, 25 mM Tris.Cl and 10 mM EDTA) and lysed by the addition of 200 μl 0.2 M NaOH/1% (w/v) SDS. Protein and genomic DNA was precipitated by the addition of 3 M potassium acetate (pH 4.8) for 5 min on ice followed by centrifugation at 12 000 g for 2 min. The supernatant, containing plasmid DNA was removed to a fresh tube and precipitated with 2 volumes of ethanol at room temperature. Plasmid DNA was pelleted at 12, 000g for 5 minutes at 4°C. Pellets were washed in 70% (v/v) ethanol and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

2.5.7.2. Caesium chloride maxi-preparations

For purification of large quantities of CDM plasmid clones, 250 mls - 1 l of culture was grown (section 2.5.7). This protocol is essentially a scaled up version of the minipreparation protocol (section 2.5.7.1.) with the addition of a caesium chloride (CsCl) density gradient purification step included to give transfection grade plasmid DNA. Pelleted cells were resuspended in 10 mls GTE buffer (see section 2.5.7.1), lysed with 20 ml 0.2 M NaOH/1% (w/v) SDS and protein and genomic DNA precipitated with 3 M potassium acetate (pH 4.8) for 10 mins on ice. Following centrifugation (10 000 g, 15 min) the supernatant was filtered through cheesecloth and DNA precipitated by the addition of 0.6 volumes of isopropanol for 10 minutes at room temperature. DNA was pelleted at 12 000 g for 15 minutes, washed with 70% (v/v) ethanol and resuspended in 3 ml TE buffer (pH 8.0). CsCl gradients were used to purify DNA. 1g CsCl was dissolved in every 1 ml of resuspended DNA. Ethidium Bromide was added to a final concentration of 740 μg/ml. Following removal of precipitate by centrifugation, the solution was

transferred to an ultracentrifuge tube and spun at 100 000 g for 16 hours. The lower of the two DNA bands in the centre of the density gradient consists of closed circular plasmid DNA, and this band was collected using a hypodermic needle. Ethidium bromide was removed by repeated extraction with NaCl saturated butan-1-ol and CsCl was removed by precipitation of the DNA with ethanol. The DNA was resuspended in TE buffer (pH 8).

2.5.8. Semi Quantitative RT-PCR

For reverse transcriptase PCR (RT-PCR) cDNA was prepared from total RNA using an Invitrogen cDNA cycle kit with oligo dT primers. Briefly, reactions were carried out in 20 µl containing 1 µg total RNA, RNase inhibitor, 5 mM dNTPs, 4 mM sodium pyrophosphate, 4 µl Invitrogen 5xRT reaction buffer and 0.5 µl AMV reverse transcriptase (RT). Reactions were carried out at 42°C for 1 hour followed by phenol chloroform extraction and ethanol precipitation. cDNA was aliquoted and frozen at -70°C until used in PCR reactions. Aliquots of cDNA were amplified by PCR as follows. To amplify FcγRIIa and FcγRIIb mRNA, a single crossreactive forward primer was used (FcγRII-FOR) with reverse primers specific for either FcγRIIa (RIIa-REV - Table 2.2) or FcγRIIb (RIIb-REV - Table 2.2). For FcγRIIb these primers flank the alternative FcγRIIb2 splice site to allow identification of different splice variants by PCR product size. PCR reactions were carried out in 50µl containing 20 pmole of each primer, 2.5 mM $MgCl_2$, 5 µl Promega Taq 10x reaction buffer and 2 units Promega Taq polymerase. PCR reactions were continued for 20 to 40 cycles with each cycle comprising 1 minute at 93°C (denaturing), 1 minute at 50°C (primer annealing) and 1 minute at 72°C (product extension). To confirm the specificity of PCR reactions, products were Southern blotted and hybridized with end labeled oligonuleotide probes (section 2.5.3.2.) specific for either FcγRIIa or FcγRIIb (Table 2.2). Further, PCR products were subcloned into the pGEM-EasyT vector (Promega) (section 2.5.8.2.) and sent for automatic sequencing. To quantify relative levels of mRNA from different samples, FcγRIIa and FcγRIIb were amplified together. At 20 cycles, formation of product was shown to be linear with levels of input cDNA (data not shown). Following 20 cycles of PCR, products were subjected to Southern blot analysis (section 2.5.8.1) and hybridized with a specific ^{32}P random

primer labeled probes (section 2.5.3.1.). Levels of cDNA levels were normalised between samples using primers specific for cyclophilin (Table 2.2).

2.5.8.1. Southern blotting PCR products

PCR products were electrophoresed as described in section 2.5.5. Gels were then soaked for 30 minutes each in denaturing buffer (1.5M NaCl, 0.5 M NaOH, pH 13), neutralising buffer (1.5 M NaCl, 1M Tris.HCl, pH7.5) and 20 x SSC. DNA was transferred to nitrocellulose (Amersham) overnight by capillary elution with 20 x SSC as transfer buffer. DNA was fixed to the nitrocellulose membrane by irradiating with ultraviolet light. Blots were hybridized with either random primer labeled double strand DNA probes (section 2.5.3.1.) or with end labeled oligonucleotide probes (section 2.5.3.2) specific for either FcγRIIa of FcγRIIb (table 2.2).

2.5.8.2. Subcloning PCR products into pGEM Easy-T cloning vector

The pGEM Easy-T cloning vector has 3'thymidine (T) overhangs to enhance the efficiency of PCR product ligation by taking advantage of the single 3' adenosine (A) added to the ends of PCR products by Taq DNA polymerase. Following electrophoresis, the PCR products were excised from the gel and purified using a Qiagen gel extraction kit. Purified PCR products were ligated into the pGEM Easy-T (50 ng/reaction) using T4 DNA ligase overnight at 4°C according to the manufacturers protocol. Ligated vector was transformed into JM109 competent cells as decribed in section 2.5.6. Transformed cells were plated onto LB plates containing, Ampicillin (100μg/ml), 0.1mM IPTG, X-Gal (40μg/ml), to allow blue/white screening. Following incubation overnight at 37°C, white colonies were picked and minipreps carried out (section 2.5.7.1.). Purified plasmids were screened for the presence of inserted PCR products by digestion with EcoRI (section 2.5.4.). Cloned PCR products were sequenced and used as probes for Southern blot hybridisations (2.5.8.1.).

Table 2.1. Antibodies

Specificity(clone/species[1])	Usage[2]	Source[3]
FcγRI mIgG1 (10.1)	IP, FACS	Calbiochem
FcγRI mIgG1 (22)	IP, FACS, X-link	Medarex
FcγRII mIgG1 (KB61)	IP, FACS, X-link	DAKO
FcγRII $mIgG_{2a}$ (IV3)	IP, FACS, X-link	Medarex
FcγRIII mIgG1 (3G8)	FACS	Medarex
CD89 mIgG1 (A3)	IP, FACS, X-link	Serotec
human IgG and subtypes	IP, FACS, X-link	Serotec
anti-mouse IgG (goat)	X-link	Sigma
Anti-human IgG (goat)	X-link	Sigma
SLP76 (sheep)	Wn, IP	Upstate
pTyr $mIgG_{2b}$(4G10)	Wn	Upstate
γ-chain (rabbit)	Wn	NEB
JNK and pJNK (rabbit)	Wn	NEB
ERK and pERK (rabbit)	Wn	NEB
p38 and pp38 (rabbit)	Wn	NEB
pAkt (rabbit)	Wn	Santa Cruz
Sos (rabbit)	Wn	Santa Cruz
Vav (rabbit)	Wn	Santa Cruz
Cbl (goat)	Wn	Santa Cruz
Grb2 (rabbit)	Wn, IP	Santa Cruz
SHIP (goat)	Wn, IP	Santa Cruz
Syk (rabbit)	Wn	Santa Cruz
Lyn (rabbit)	Wn	Santa Cruz
PLC (rabbit)	Wn	Santa Cruz
p85 (rabbit)	Wn	Santa Cruz
Shc (rabbit)	Wn	Santa Cruz
Shc (mouse)	Wn, IP	Santa Cruz
RasGAP (mouse)	Wn	Santa Cruz
anti-mouse: FITC	FACS secondary Abs	Sigma
anti-mouse:HRP	Wn secondary Ab	Amersham
anti-rabbit:HRP	Wn secondary Ab	Amersham
anti-sheep:HRP	Wn secondary Ab	Upstate
anti-goat:HRP	Wn secondary Ab	Sigma

Key for Table 2.1 (see previous page)

1 clone number and/or species in which antibody was raised.

2 IP; immuneprecipitation. FACS; fluorescent activated cell scanning. X-link; crosslinking studies. Wn; western blotting.

3 manufacturer

Amersham, Amersham, UK

Serotec, Oxford, UK

Sigma, Poole, UK

Upstate, Upstate Biotechnology, Lake Placid, USA

Santa Cruz, Santa Cruz Biotechnology, Sta Cruz, CA, USA

Medarex, NJ, USA

NEB, New England Biolabs, MA, USA

DAKO Ltd, Cambridgeshire, UK

Table 2.2. Oligonucleotide primers

Primer	Description	Sequence
II-FOR	FcγRII forward primer	GCACAGGAAACATAGGCTACACG
IIa-REV	FcγRIIa reverse primer	GGTATCTTCTTAGAAAGTCCC
IIb-REV	FcγRIIb reverse primer	GGTGATTGTGTTCTCAGCCCC
IIa-probe	FcγRIIa probe	CCTCCCAACGACCATGTCAACAG
IIb-probe	FcγRIIb probe	GATGAGGCTGATAAAGTTGGGGC
Cyc-FOR	cyclophilin forward primer	GGTGACTTCACACGCCATAATG
Cyc-REV	cyclophilin reverse primer	GAGTTGTCCACAGTCGGAGATG

Chapter 3

FcγRI activates SH2 containing inositol phosphatase (SHIP)

3.1. Introduction

Receptors for immunoglobulins (Ig) play a pivotal role linking the humoral and cellular arms of the immune system and thereby regulating the immune response to antigen. Each immunoglobulin class is recognised by a family of low and high affinity receptors (Hulett & Hogarth, 1994, Ravetch & Kinet, 1991). Thus, IgG is recognised by Fcγ receptors and three different subclasses of receptors (FcγRI, FcγRII and FcγRIII) have been defined on the basis of their structure and affinities for IgG. These receptors (except FcγRIIIb) comprise integral type I membrane glycoproteins and all possess similar overall structure. However, the cytoplasmic tails of the various receptors are highly divergent and these specify recruitment of different signalling pathways.

In common with many immunoreceptors, FcγRs couple to the activation of signal cascades via clustering of cytoplasmic immunoreceptor tyrosine activation motifs (ITAM) (Cambier *et al.*, 1995, Cambier, 1995). These ITAM motifs are generally found on receptor associated accessory molecules such as the γ-chain, which associates with FcγRI, FcγRIII and FcεRI or with the α and β chains of the BCR. Occasionally, as is the case for FcγRIIa, the ITAM motif can be found in the cytoplasmic tail of the receptor itself. Aggregation of Fc receptor associated ITAM motifs by immune complex stimulates the activity of associated Src family kinases which phosphorylate key tyrosine residues within the ITAM motif. These phosphorylated ITAMs comprise docking sites for SH2 domain containing signalling molecules, including the tyrosine kinase Syk, and adapter proteins, which propagate signals (Agarwal *et al.*, 1993, Cambier & Johnson, 1995, Duchemin & Anderson, 1997).

The recruitment and activation of Syk to activated ITAMs is critical for the activation of multiple key signalling pathways activated by Fc receptors (Crowley *et al.*, 1997).

Probably the most studied of these Syk dependent pathways is the activation of phospholipase C (PLC) to generate the second messengers, inositol trisphoaphate ($InsP_3$) and diacylglycerol (DAG), and resultant calcium transients (Liao, Shin & Rhee, 1992, Rankin *et al.*, 1993). Following receptor aggregation, PLCγ translocates to the plasma membrane where it becomes activated in a PI 3-kinase dependent manner (discussed in section 1.2.3.5) (Falasca *et al.*, 1998, Fluckiger *et al.*, 1998). Following activation, PLCγ catalyses the production of $InsP_3$ and DAG from its membrane associated lipid substrate, PIP_2. Production of $InsP_3$ is responsible for calcium release from intracellular stores and subsequent calcium influx through the store operated calcium channel termed I_{CRAC} (Hoth & Penner, 1992).

Alongside ITAM bearing receptors, responsible for cellular activation, a new family of inhibitory immuno-receptors has recently been described (Daeron, 1996). These inhibitory receptors become activated when co-clustered with activating receptors and serve to block, or modulate, cellular responses to the activating signal. The inhibitory properties of these receptors are mediated through a cytoplasmic immunoreceptor tyrosine based inhibitory motif (ITIM) (Muta *et al.*, 1994a, Amigorena *et al.*, 1992b) which is closely related to the ITAM motif. Following receptor clustering, the ITIM motif becomes phosphorylated on a key tyrosine residue, which then serves to recruit SH2 containing inhibitory signalling molecules. Two functionally distinct inhibitory pathways have thus far been described for ITIM bearing receptors. Initially, the phosphorylayed ITIM motif was described as binding and activating, *in vitro*, the SH2 containing tyrosine phosphatases, SHP-1 (Dambrosio *et al.*, 1995) and SHP-2 (Marengere, 1996). By activating tyrosine phosphatases, inhibitory receptors are able to block tyrosine kinase dependent activation. Such a mechanism appears to account for the inhibitory properties of, among others, the ITIM bearing killer inhibitory receptors (KIRs) and CD22 (Unkeless & Jin, 1997). However, this mechanism does not seem to account for the inhibitory properties of the ITIM bearing Fcγ receptor, FcγRIIb. Here, inhibition appears to centre round the recruitment of the SH2-containing inositol 5'phosphatase (SHIP) to the phosphorylated ITIM (Scharenberg & Kinet, 1996).

The inhibitory properties of FcγRIIb have been extensively studied for the B-Cell receptor (BCR) in B lymphocytes and FcεRI in Mast cells (section 1.2.8.4 and 1.2.8.5). Co-ligation of FcγRIIb with either the BCR or FcεRI, results in the recruitment of SHIP to the plasma membrane where it dephosphorylates PIP_3, the lipid product of PI 3-kinase. PIP_3 acts to target specific signalling molecules, including PLCγ (Falasca *et al.*, 1998) and Tec family kinases (Scharenberg *et al.*, 1998), to the plasma membrane. Thus, by dephosphorylating PIP_3, SHIP is able to uncouple receptors from a specific subset of activatory pathways (Bolland *et al.*, 1998, Scharenberg & Kinet, 1996). A major target of this inhibitory pathway is PLCγ, which under inhibitory conditions fails to generate the sustained production of $InsP_3$ required for calcium influx. Interestingly, FcγRIIb co-ligation with the BCR (Muta *et al.*, 1994a) or FcεRI (Ono *et al.*, 1996) does not block calcium release from intacellular stores and also leaves tyrosine phosphorylation largely unaffected . Thus, unlike SHP-1 and SHP-2 mediated inhibition, SHIP is able to modulate, rather than entirely block, the cellular response to an activating signal.

The human high affinity receptor, FcγRI, contains no known signalling motif or homology with other immune system receptors (Allen & Seed, 1989). In IFN-γ primed U937 cells, FcγRI associates non-covalently with the γ chain (Ernst *et al.*, 1993, Ra *et al.*, 1989, Scholl & Geha, 1993) which contains an ITAM motif (Cambier, 1995). Aggregation of FcγRI by surface immune complexes results in clustering of the associated γ chain and activation of the nonreceptor tyrosine kinases, Lyn and Syk, to initiate signal transduction as evidenced by tyrosine phosphorylation events (Scholl, Ahern & Geha, 1992, Rankin *et al.*, 1993) and tyrosine kinase dependent calcium transients (Davis *et al.*, 1994). In these cells, FcγRI aggregation results in release of calcium from intracellular stores which is not coupled with calcium entry (Davis *et al.*, 1994). Further, FcγRI fails to activate PLCγ and thus $InsP_3$ production. As the pattern of calcium transients and failure to activate PLCγ following FcγRI aggregation resembles the signalling phenotype observed when coligating FcγRIIb with the BCR or FcεRI, I decided to examine the possible involvement of SHIP in regulating FcγRI signalling.

Here, I show that SHIP is present in the membrane fraction of IFN-γ treated cells and, after FcγRI aggregation, SHIP is transiently tyrosine phosphorylated. Further, the SH2

domain containing adapter protein, Shc, also became tyrosine phosphorylated, translocated to the membrane fraction and became tightly associated with SHIP. Finally, SHIP appeared to be recruited to immune complexes following FcγRI aggregation possibly through interactions with the SH2 containing adapter molecule, Shc.

3.2. Results

3.2.1. SHIP becomes tyrosine phosphorylated in response to FcγRI aggregation

To determine whether SHIP was expressed in IFN-γ stimulated U937 cells, lysates were examined by Western blotting with anti-SHIP antibodies (section 2.3.6). The mouse macrophage cell line P388D1 was used as a positive control. SHIP was detectable as a 145 kDa protein in both cell types (Figure 3.1A). This antibody, but not goat IgG, was also found to immune precipitate SHIP from U937 cell lysates (Figure 3.1B). Further, SHIP immune precipitates contained readily detectable Ins(1,3,4,5)P_4 5-phosphatase activity whereas no activity was detectable in control immune precipitates (Figure 3.1C). I conclude that SHIP is expressed in IFN-γ stimulated U937 cells.

To examine whether SHIP was involved in FcγRI mediated signalling pathways, plasma membrane preparations of cells at various time points following receptor aggregation were analysed by Western blot. Probing with anti-phosphotyrosine antibodies (section 2.3.6.1) revealed that a protein of the correct size for SHIP, is transiently phosphorylated following FcγRI aggregation, with peak phosphorylation occurring after 1-2 minutes (Figure 3.2A). Analysis of blots with anti-SHIP antibodies indicates that levels of membrane bound SHIP remain unchanged in response to crosslinking, ruling out membrane translocation as a regulatory mechanism. To assess the effect of tyrosine phosphorylation on the enzymatic activity of SHIP, 5' phosphatase activity was assayed in SHIP immune precipitates both before and two minutes after receptor crosslinking. In agreement with the results of other groups (Damen *et al.*, 1996), tyrosine

phosphorylation was not seen to significantly affect the activity of SHIP *in vitro* (Figure 3.2B).

3.2.2. FcγRI aggregation induces the association of Shc with SHIP

SHIP appears to be constitutively associated with the plasma membrane; its site of action and activity is apparently independent of tyrosine phosphorylation. It was therefore decided to immune precipitate SHIP over a time course following FcγRI aggregation and blot for associated tyrosine phosphorylated proteins which might play a regulatory role. Following stimulation, SHIP was seen to transiently associate with two tyrosine phosphorylated proteins of around 52 and 60 kDa. I identified these bands to be two isoforms of the adapter protein, Shc (Figure 3.3). The comparable time course of tyrosine phosphorylation of both Shc and SHIP together with the time course of Shc association with SHIP suggests this interaction to be tyrosine phosphorylation dependent.

To assess the interaction between SHIP and Shc more accurately, I immune precipitated Shc over a more extensive time course following FcγRI aggregation. SHIP became detectable in Shc immune precipitates within 1 minute of receptor aggregation, remained until 5 minutes but had disappeared after 15 minutes (Figure 3.4A). Probing for phosphotyrosine revealed that SHIP was always tyrosine phosphorylated in Shc immune precipitates (Figure 3.4A). Close examination of these anti-phosphotyrosine blots of Shc immune precipitates revealed the presence of a second transiently tyrosine phosphorylated protein of around 70 kDa associated with Shc . This protein was identified to be the tyrosine kinase, Syk (Figure 3.4A). Unlike the association of Shc with SHIP, Syk appears to be pre-associated with Shc, and is rapidly tyrosine phosphorylated after receptor crosslinking. Peak phosphorylation was observed 1 minute after crosslinking which is faster than the observed association of Shc with SHIP. Further analysis of SHIP immune precipitation blots revealed the presence of a transiently tyrosine phosphorylated protein of the correct size for Syk associating with SHIP (Figure 3.3 - indicated by arrow). This suggests that a trimolecular complex containing SHIP, Shc and Syk may form transiently in response to FcγRI aggregation.

To confirm the interaction of SHIP with Shc, 5'phosphatase activity associated with Shc was assayed prior to FcγRI crosslinking and two minutes after crosslinking. No measurable 5' phosphatase activity was observed in Shc immune precipitates from uncrosslinked cells, whereas two minutes following crosslinking, activity was readily detectable (Figure 3.4B). This process occured independently of the activation of PI 3-kinase or phospholipase D (Melendez *et al.*, 1998b) as wortmannin (100 nm), LY294002 (250 μM), butan-1-ol (0.3% v/v) and butan-2-ol (0.3% v/v) did not influence the association of SHIP with Shc (data not shown). Analysis of membrane fractions also revealed that Shc transiently translocates from the cytosol to the plasma membrane (Figure 3.5). Peak levels were seen two minutes after crosslinking, concomitant with peak levels of SHIP tyrosine phosphorylation, suggesting this translocation may be dependent on an interaction with SHIP. Levels of cytosolic Shc were seen to decrease following FcγRI crosslinking, concomitant with the increase of Shc in the plasma membrane fraction (data not shown), indicating that Shc is translocating from the cytosol.

3.2.3. SHIP is detectable in immune complexes following receptor stimulation

To determine whether SHIP and Shc associate with FcγRI signalling complexes membrane bound immune complexes were precipitated with protein G agarose. Western blot analysis indicates that both SHIP and Shc are detectable 2 minutes after receptor aggregation (Figure 3.6A). Surprisingly, levels were seen to remain elevated 15 minutes post aggregation, when SHIP phosphorylation has returned to near basal levels. SHIP and Shc were not present in control immune precipitations where no sheep anti-human IgG was included. The coincident appearance of SHIP and Shc is consistent with a model in which SH2 domain interactions between SHIP and Shc are involved in recruitment to signalling complexes. The presence of SHIP in these immune complexes was confirmed by a substantial increase in 5'phosphatase activity in this fraction after FcγRI aggregation (Figure 3.6B).

3.3. Discussion

I have shown that SHIP, a key inhibitory regulator of immune cells, is present in IFN-γ primed U937 cells. Following aggregation of FcγRI by the formation of surface immune complexes, SHIP is transiently tyrosine phosphorylated and associates with the adapter protein, Shc.

Previous work from this group has shown that aggregation of FcγRI in these cells activates a novel pathway which involves the sequential activation of tyrosine kinases, PI3-kinases, phospholipase D and sphingosine kinase (Melendez *et al.*, 1998b). The resultant calcium transients are short lived being derived entirely by release from internal stores, with no element of calcium influx (Davis *et al.*, 1994). Interestingly, activation of phospholipase C (PLC) could not be observed following FcγRI aggregation (Melendez *et al.*, 1998a). The PLC pathway is intact in these cytokine primed cells as PLC can be activated by other receptors, including FcγRIIa. Under these circumstances, activation of PLC is associated with calcium transients where calcium influx is observed (Melendez *et al.*, 1998a).

My finding here that SHIP is activated following FcγRI aggregation provides an explanation for these observations. Increasing evidence supports a role for PIP_3 as a positive regulator of calcium influx. By hyrolysing PIP_3, SHIP prevents the translocation of key signalling proteins to the plasma membrane. In particular, the Tec family kinases, which bind to PIP_3 via their PH domains, have been identified as potential targets of a SHIP mediated inhibitory signal (Bolland *et al.*, 1998, Scharenberg *et al.*, 1998). By preventing the translocation of Tec family kinases SHIP might prevent activation of downstream targets, such as PLCγ1, which is required for sustained calcium influx. Preventing the translocation and activation of PLCγ1, prevents sustained production of $InsP_3$ which is required to activate store mediated calcium influx (Fluckiger *et al.*, 1998). Such a mechanism has been proposed for the action of SHIP in B-cells and mast cells where SHIP is recruited by FcγRIIb (Scharenberg *et al.*, 1998).

The mechanism by which FcγRI activates SHIP remains unclear as FcγRI has not been reported to associate with any ITIM bearing receptors. For signal transduction, FcγRI uses the γ chain which only features an activation motif or ITAM. However, recent work has shown that the cytoplasmic tail of the Granulocyte Colony-Stimulating Factor Receptor can recruit SHIP independent of an ITIM through a mechanism involving Shc and the formation of Shc/SHIP complexes (Hunter & Avalos, 1998). The finding that the adapter molecule Shc forms a tight association with SHIP following FcγRI aggregation provides a mechanism to link the FcγRI:γ chain complex to SHIP. Formation of this complex was apparently tyrosine phosphorylation dependent and the tyrosine kinase Syk appeared to associate with Shc. This suggests that, consistent with previous reports (JabrilCuenod *et al.*, 1996, Crowley, Harmer & DeFranco, 1996), Syk is involved in the phosphorylation of Shc and SHIP.

SHIP itself was found in the plasma membrane and, when immunoprecipitated from the cell, SHIP was constitutively active. This implies that tyrosine phosphorylation itself does not regulate enzyme activity but rather may play a role to target the enzyme to the correct adapter molecule such as Shc through SH2 domain interactions. It also suggests that there may be negative regulators of the enzyme to prevent activation in the absence of SH2 domain interactions.

Figure 3.1 SHIP 5'inositol phosphatase is expressed in monocytic U937 cells.

(A) Protein extracts (100 μg/lane) from IFN-γ primed U937 cells and the mouse macrophage cell line, P338D1, were analysed by Western blot using anti-SHIP antibodies as described. (B) Lysates from IFN-γ primed U937 cells were immunoprecipitated with either anti-SHIP conjugated protein-G agarose or control unconjugated protein-G agarose (control) as described in the materials and methods (section 2.3.4.1). Immune precipitates were then analysed by Western blot (section 2.3.6). (C) SHIP immune precipitates (Anti-SHIP) and control immune precipitates (Control) were subjected to 5'inositol phosphatase assays as described previously (section 2.4.2). Background control assays were carried out in the absence of any immune precipitate (Blank). Data are the mean ± standard deviation (SD) of triplicate measurements derived from four separate experiments.

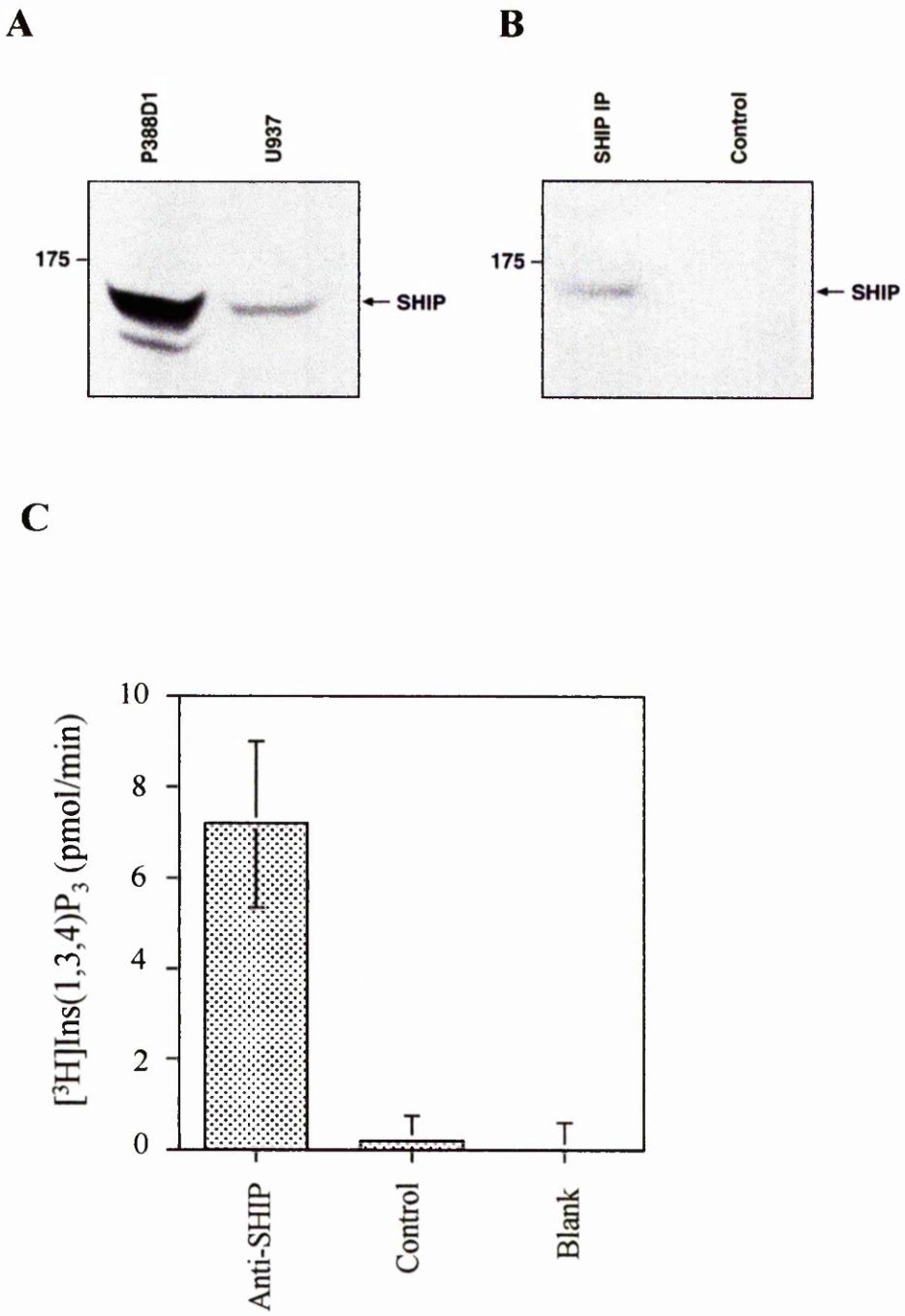
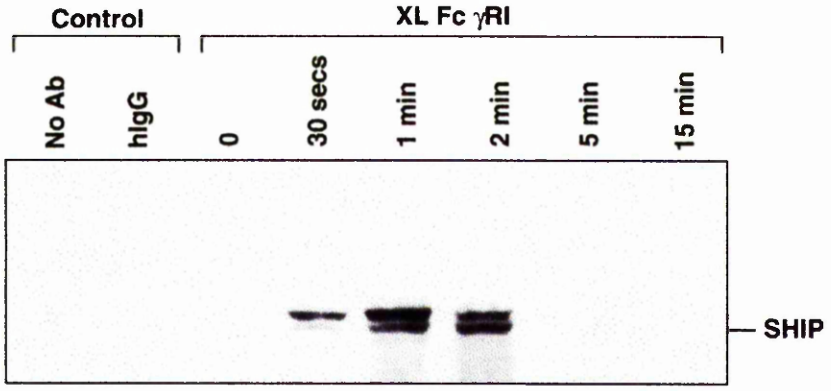

A
P388D1
U937
175
SHIP
B
SHIP IP
Control
175
SHIP
C
[3H]Ins(1,3,4)P3 (pmol/min)
10
8
6
4
2
0
Anti-SHIP
Control
Blank

Figure 3.2 Membrane associated SHIP becomes tyrosine phosphorylated following FcγRI cross linking.

(A) IFN-γ primed U937 cells were loaded with monomeric hIgG at 4°C and IgG occupied FcγRI was aggregated with sheep anti-hIgG as described in the materials and methods (section 2.3.1.1). Cells were warmed to 37°C for the times specified. Membrane fractions (30 μg/lane) were isolated (section 2.3.8) and analysed by Western blot. Phosphotyrosine-containing proteins were detected using anti-phosphotyrosine (4G10) antibody. Membranes were stripped and re-probed with anti-SHIP antibodies. Control lanes contain membrane fractions from cells incubated with either no antibodies (No Ab), or just hIgG (hIgG). (B) Cells were incubated with either hIgG alone (No XL) or crosslinked for 2 minutes at 37°C (XL). Lysates were immune precipitated with either anti-SHIP antibodies (SHIP) or control goat IgG (Control). Immune precipitates were subjected to 5'inositol phosphatase assays as described in section 2.4.2. Background control assays were carried out in the absence of any immune precipitate (Blank). Data are the mean ± SD of triplicate measurements derived from three separate experiments.

A


Control
XL Fc γRI
No Ab
hIgG
0
30 secs
1 min
2 min
5 min
15 min
SHIP

B

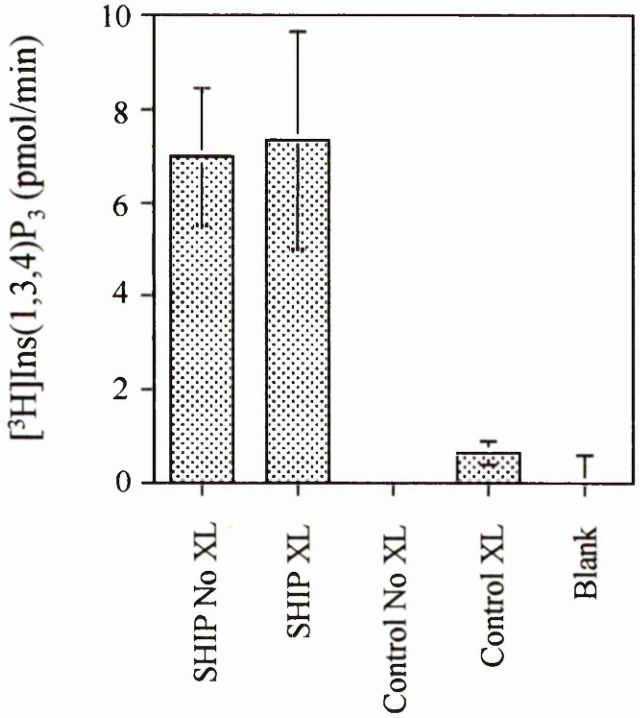

[3H]Ins(1,3,4)P3 (pmol/min)
10
8
6
4
2
0
SHIP No XL
SHIP XL
Control No XL
Control XL
Blank

Figure 3.3 SHIP associates with tyrosine phosphorylated Shc following FcγRI cross linking.

(A) Western blot analysis of SHIP immunoprecipitates probed with antiphosphotyrosine (4G10) antibody. FcγRI was aggregated for 30 sec, 2 min and 10 min as indicated. Cells loaded with hIgG but with no addition of sheep anti-hIgG are used as control. SHIP immune precipitates were analysed by Western blot using anti-phosphotyrosine mAb 4G10 (upper), anti-SHIP antibody (middle), and anti-Shc antibody (lower).

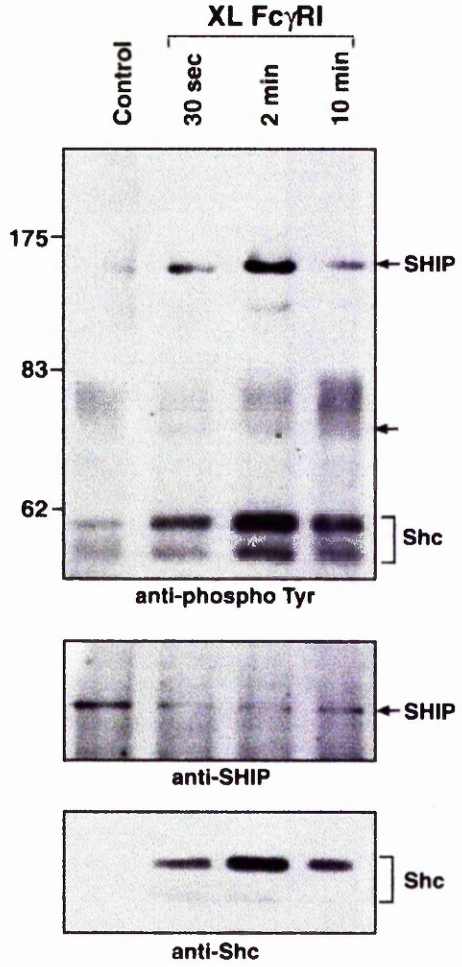
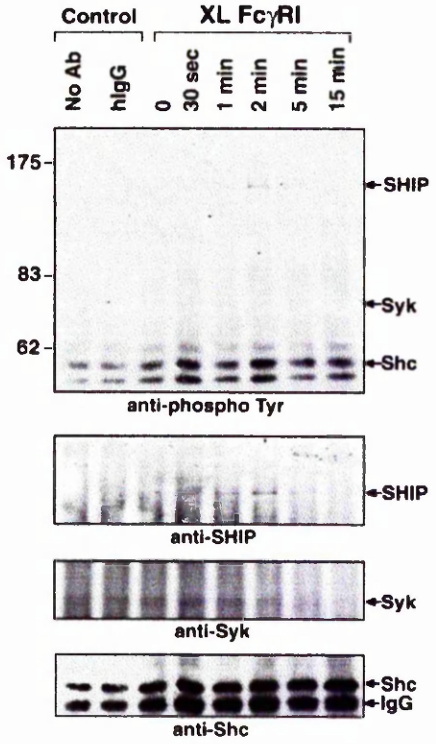

XL FcγRI
Control
30 sec
2 min
10 min
175
83
62
SHIP
Shc
anti-phospho Tyr
SHIP
anti-SHIP
Shc
anti-Shc

Figure 3.4 Shc associated SHIP has 5'inositol phosphatase activity.
(A) Western blot analysis of Shc immunoprecipitates. FcγRI was aggregated for 0, 30 sec, 1 min, 2 min, 5 min and 15 min as indicated. Cells with no addition of hIgG or sheep anti-hIgG (No Ab) and cells loaded with hIgG but no sheep anti-hIgG (IgG) were included as controls. Shc immune precipitates were analysed by Western blot using anti-phosphotyrosine antibody (upper). Blots were stripped and reprobed with anti-SHIP, anti-Syk and anti-Shc antibodies (lower panels) as indicated. The position of the precipitating anti-Shc antibody (IgG) is indicated on anti-Shc and anti-phosphotyrosine blots. This antibody is detected by the HRP-conjugated secondary antibody alone. (B) 5'-inositol phosphatase assay of Shc immune precipitates. Shc was immune precipitated from either uncrosslinked cells (Shc No XL) or FcγRI crosslinked cells (Shc XL). Lysates were also precipitated with non-reactive serum as control (Control No XL and Control XL). Background control assays were carried out in the absence of any immune precipitate (Blank). Data are the mean ± SD of triplicate measurements derived from three separate experiments.

A


Control
XL FcγRI
No Ab
hIgG
0
30 sec
1 min
2 min
5 min
15 min
175
83
62
SHIP
Syk
Shc
anti-phospho Tyr
SHIP
anti-SHIP
Syk
anti-Syk
Shc
IgG
anti-Shc

B

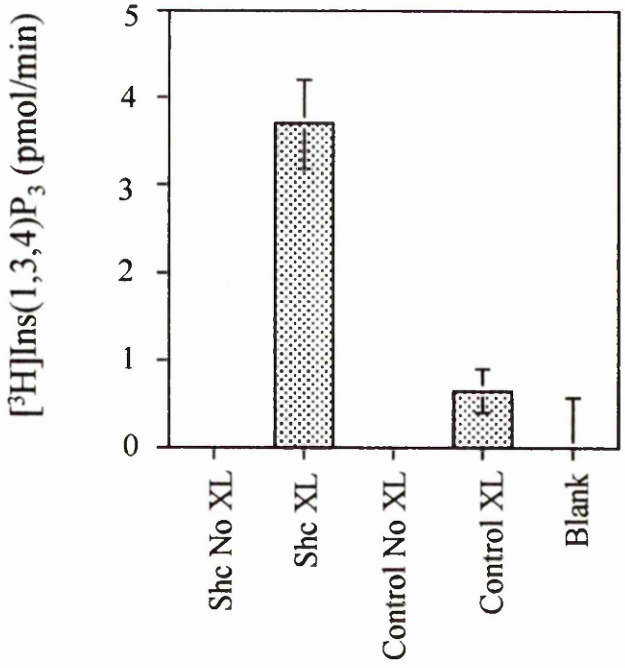

[3H]Ins(1,3,4)P3 (pmol/min)
5
4
3
2
1
0
Shc No XL
Shc XL
Control No XL
Control XL
Blank

Figure 3.5 FcγRI aggregation induces the translocation of Shc to the plasma membrane

Western blot analysis of membrane fractions. FcγRI was aggregated for 30 secs, 1 min, 2 min, 5 min and 15 min. Membrane fractions (30μg/lane) were analysed by Western blot, probing with either anti-SHIP antibody (upper) or anti-Shc antibody (lower). Cells loaded with hIgG but no sheep anti-hIgG (IgG) was included as a control.

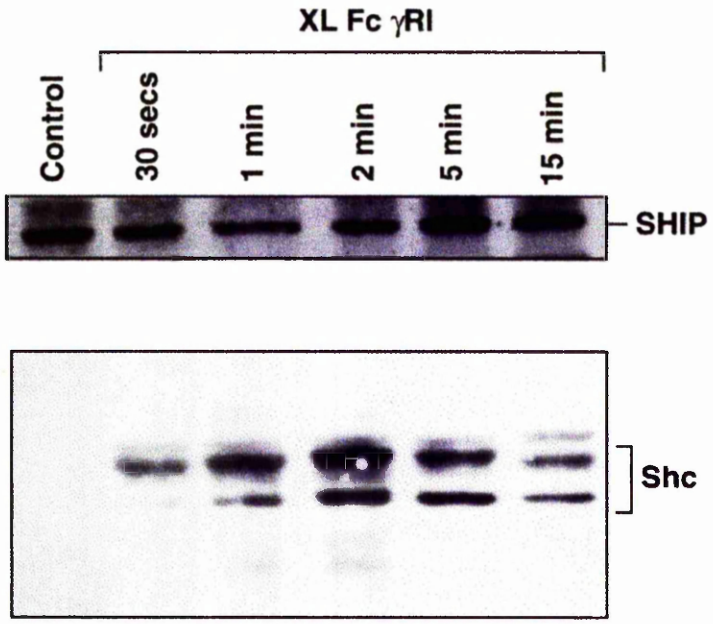
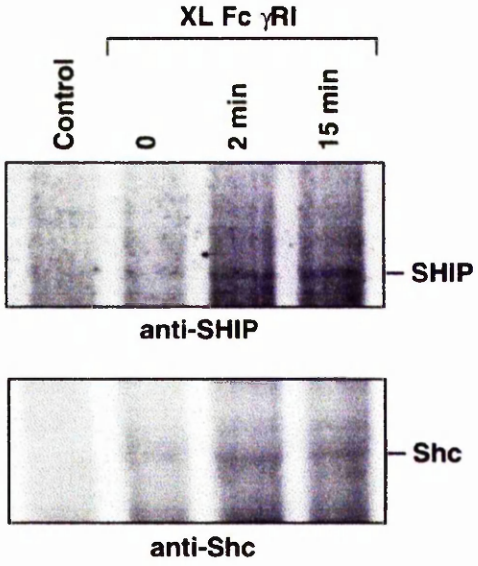

XL Fc γRI
Control
30 secs
1 min
2 min
5 min
15 min
SHIP
Shc

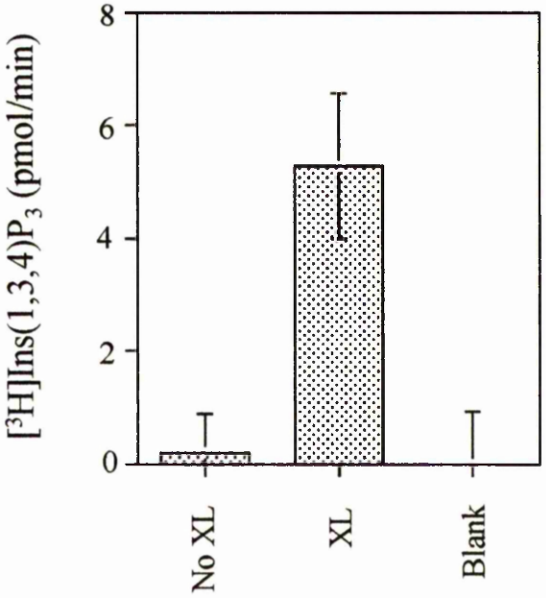
Figure 3.6 SHIP and Shc are detectable in immune complexes following receptor stimulation.

(A) FcγRI was aggregated for 30 sec, 2 min and 15 min as indicated. FcγRI based immune complexes were precipitated from membrane preparations by the addition of protein-G agarose. SHIP and Shc were detected by Western blot using anti-SHIP (upper) and anti-Shc (lower) antibodies. Cells loaded with hIgG alone act as an immune precipitation control. (B) 5'-inositol phosphatase assay of immune complex precipitates. Immune complexes were precipitated with protein-G agarose from uncrosslinked cells (No XL) or FcγRI crosslinked (XL) cells (37°C for 2 min). Background control assays were carried out in the absence of any immune precipitate (Blank). Data are the mean ± SD of triplicate measurements representative of three separate experiments.

A


XL Fc γRI
Control
0
2 min
15 min
SHIP
anti-SHIP
Shc
anti-Shc

B


[3H]Ins(1,3,4)P3 (pmol/min)
8
6
4
2
0
No XL
XL
Blank

Chapter 4

Differentiation of U937 cells with dbcAMP induces the expression of FcγRIIb

4.1. Introduction

Macrophages play a key role in the coordination of inflammation. These cells must be activated to combat infection and kill abnormal cells such as virus infected cells and cancer cells. However, activation must be tightly regulated as effector functions of these cells, such as degranulation, the respiratory burst and generation of reactive oxygen species, can lead to tissue destruction and disease processes. It is important therefore to balance activating and inhibitory signals to precisely control the state of macrophage activation.

Fcγ receptors play a key role in the regulation of macrophage activation and also in the coordination of inflammation (section 1.3). Thus aggregation of Fcγ receptors by immune complexes leads to antigen internalisation, activation of various cytotoxic effector functions and production of inflammatory cytokines such as TNF-α (section 1.1.8). While the specific signalling pathways linking Fcγ receptors to these effector functions are becoming clearer, less is known concerning potential inhibitory pathways which switch off, or prevent incorrect activation of these cells.

Recent studies using mouse knockout models have indicated the importance of Fcγ receptors in mediating tissue damage in auto-immune and inflammatory disease models (Clynes *et al.*, 1999, Clynes *et al.*, 1998, Yuasa *et al.*, 1999, Ravetch & Clynes, 1998). Mice deficient for the ITAM containing γ-chain are completely protected from immune complex mediated inflammatory injury. Conversely, mice lacking the inhibitory ITIM bearing receptor, FcγRIIb, show enhanced susceptibility to, and severity of, disease in multiple model systems. Further, macrophages isolated from mice lacking FcγRIIb show enhanced Fcγ receptor mediated calcium signalling, phagocytosis and inflammatory

cytokine synthesis (Yuasa *et al.*, 1999, Clynes *et al.*, 1999). These studies highlight the importance of both positive and negative signalling pathways in the correct regulation of murine macrophage activation by immune complexes.

Previous work in our laboratory has defined a model system using human U937 monocyte cells for studying Fcγ receptor mediated signalling pathways. Here we have demonstrated that the signalling pathway used by FcγRI to mobilise calcium is dependent on the differentiation state of the cell (Melendez *et al.*, 1998a). Further, we have demonstrated that this switch is dependent on the accessory molecule recruited by FcγRI. Thus, in IFN-γ primed U937 cells, FcγRI couples via the γ-chain to PLD, sphingosine kinase and calcium store release (Chapter 6) (Melendez *et al.*, 1998a). Conversely, in dbcAMP cells FcγRI couples via FcγRIIa to phospholipase C, calcium store release and calcium influx (Chapter 6) (Melendez *et al.*, 1998a).

Differences in calcium signalling and the nature of phospholipase enzyme activation between the differentiation states prompted us to look for inhibitory signalling pathways activated by Fcγ receptors (see Chapter 3). As FcγRIIb has been shown to modulate calcium signalling and phospholipase C activation by ITAM receptors in B-cells and mast cells (Ono *et al.*, 1996, Fluckiger *et al.*, 1998, Bolland *et al.*, 1998), it seemed possible that similar mechanisms may regulate macrophages. As FcγRIIb expression has not been demonstrated previously in human macrophages or U937 cells, the first step was to determine whether this inhibitory receptor was expressed and under what conditions.

Here, although undifferentiated U937 cells do not express cell surface FcγRIIb, differentiation of these cells to a more macrophage phenotype with dbcAMP induces the expression of this inhibitory IgG receptor. In contrast, priming cells with IFN-γ does not induce expression of FcγRIIb. Further, in dbcAMP differentiated cells, FcγRIIb is capable of recruiting SHIP in response to co-aggregation with the activating IgG receptor, FcγRIIa. This data suggests that this receptor may act by limiting, but not blocking, specific signalling pathways activated by FcγRIIa. These findings delineate a potential novel role for FcγRIIb in the modulation of immune complex mediated activation of human macrophages.

4.2. Results

4.2.1. DbcAMP upregulates the expression of FcγRIIa and induces the expression of FcγRIIb

Changes in the expression of various subclasses of FcγRII by U937 cells in response to differentiation with either IFN-γ or dbcAMP were determined using a combination of Northern blotting, RT-PCR, FACS and Western blot analysis.

Total RNA was extracted from U937 cells at various timepoints following treatment with either IFN-γ or dbcAMP and Northern blots prepared as decribed in chapter 2 (sections 2.5.1 and 2.5.2). Hybridisation of blots with an FcγRII specific cDNA probe revealed a 2.6 kb transcript and a 1.5 kb transcript detectable at all timepoints tested following both IFN-γ and dbcAMP treatment (Figure 4.1). FcγRIIa is reported to give rise to both a 2.6 kb transcript and a 1.5 kb transcript which arise from differential polyadenylation (Brooks *et al.*, 1989). The size of the FcγRIIb transcript is also 1.5 - 1.6 kb (Brooks *et al.*, 1989). The nucleotide sequences for FcγRIIa and FcγRIIb are very similar and the cDNA probe was unable, under the conditions used here, to distinguish between the two transcripts. IFN-γ treatment lead to an increase in the 2.6 kb transcript specific for FcγRIIa but only a very small increase in the 1.5 kb transcript (FcγRIIa or FcγRIIb). Treatment with dbcAMP however leads to a dramatic rise in the levels of both the 2.6 kb and 1.5 kb transcripts within 6 hours and these remained substantially elevated even 48 hrs after treatment. Equal levels of 18S and 28S ribosomal RNA confirmed equal loading of total RNA (Figure 4.1 - bottom panel).

RT-PCR was used to determine the nature of the transcripts induced after IFN-γ or dbcAMP differentiation. cDNA was generated using oligo dT primers and AMV reverse transcriptase (section 2.5.8). For PCR, a single forward primer, crossreactive for both FcγRIIa and FcγRIIb was used in conjunction with reverse primers that were designed to be specific for either FcγRIIa or FcγRIIb (see Table 2.2). For FcγRIIa, these primers were

designed to generate a 719 bp product. For FcγRIIb, the reverse primer was designed that was specific for FcγRIIb and which lay downstream of a splice site. Thus, the primers flank a splice site which gives rise to the splice variants FcγRIIb1 or FcγRIIb2, which has a shorter C-terminal domain. Thus, the RT-PCR product for FcγRIIb1 would be 289 bp in length whereas that for FcγRIIb2 would be 232 bp in length (Figure 4.2). To quantify relative changes in the expression of specific FcγRII transcripts by RT-PCR, it was necessary to optimize the PCR conditions and cycle number to ensure a linear relationship existed between the amount cDNA template added to the PCR reaction and the amount of PCR product produced. As linearity between template and product was only maintained at low cycle numbers, it was necessary to Southern blot PCR products and hybridise blots with a crossreactive ^{32}P labelled FcγRII cDNA probe (section 2.5.8.1). The bands could then be quantified by comparing the relative hybridisation intensity between samples, as measured by quantitative phosphorimaging.

Products for both FcγRIIa, at around 700 bp, and FcγRIIb at around 300 bp, were detectable in untreated U937 cells (Figure 4.3). Differentiation with IFN-γ for 6 and 12 hours induced no significant change in expression of either FcγRIIa or FcγRIIb. Differentiation with dbcAMP however induced a massive upregulation of both FcγRIIa and FcγRIIb. Thus, levels of the FcγRIIa PCR product were increased 10 fold 24 hours after dbcAMP addition and 20 fold after 48 hours. For FcγRIIb, levels of the PCR product was increased 20 fold after 24 hours and over 50 fold at 48 hours (Figure 4.3).

These PCR products were positively identified as fragments of FcγRIIa and FcγRIIb in two ways. Firstly, oligonucleotide primers were designed that lay internal to the amplified fragment and were specific for either FcγRIIa or FcγRIIb (Table 2.2). These were ^{32}P end labeled and hybridised to Southern blots of PCR products (data not shown). The FcγRIIa specific probe hybridised only to the 700 bp fragment. The FcγRIIb specific probe hybridised only to the 300 bp fragment. Secondly, both PCR fragments were subcloned into the pGEMeasyT plasmid (Promega) and sequenced. Sequence analysis positively identified the 700 bp product as the expected fragment of FcγRIIa cDNA. The 300 bp fragment was identified as a fragment of FcγRIIb1 cDNA

As FcγRIIa and FcγRIIb have almost identical extracellular domains (section 1.1.3.2) (Brooks *et al.*, 1989), all mAbs available for fluorescent labeling of cells, with the exception of IV3, crossreact with both isoforms. The mAb IV3 (Medarex) however has been reported to only recognise FcγRIIa (Maresco *et al.*, 1999). FACS analysis (section 2.2.2.1) was carried out using mAb IV3 to determine whether FcγRIIa surface expression was upregulated by dbcAMP differentiation in response to the observed increase in mRNA expression (Figure 4.4A). In agreement with Northern and RT-PCR data, cell surface expression of FcγRIIa was upregulated by differentiation with dbcAMP for 48 hours (Figure 4.4A.). No change in cell surface expression of FcγRIIa was observed following IFN-γ treatment. Two additional antibodies, KB61 (Pulford *et al.*, 1986, Greenman *et al.*, 1991) and AT10, recognise both FcγRIIa and FcγRIIb. FACS analysis with either antibody revealed an identical pattern (Figure 4.4B and data not shown).

In addition, Western blot analysis was performed to identify FcγRIIb in cell lysates. The mAb clone IIAD2 (a gift from Professor Jurgen Frey, University of Bielefeld, Germany) recognises the intracellular tail of human FcγRIIb and shows no crossreactivity with FcγRIIa (Weinrich *et al.*, 1996). Western blots were prepared using cell lysates from untreated U937 cells or cells differentiated with either IFN-γ (24 hours) or dbcAMP (48 hours). Cell lysate from the human EDR B-cell line, known to express FcγRIIb, was included on blots as a positive control. Probing blots with IIAD2 revealed a band of the correct molecular size for FcγRIIb in all U937 cell lysates. This FcγRIIb immunoreactive band was specifically upregulated by dbcAMP (Figure 4.5A). Levels of FcγRIIb detected in lysates of cells differentiated for 48 hours with dbcAMP were comparable to levels seen in the EDR B-cell line positive control.

To test whether FcγRIIb was expressed at the cell surface in either IFN-γ or dbcAMP cells, total surface FcγRII was immune precipitated. To do this, intact cells were labeled with the pan-FcγRII mAb, KB61, followed by extensive washing to remove unbound antibody. Following cell lysis, KB61 bound FcγRII was immune precipitated and precipitates were subjected to Western blotting with mAb IIAD2. Figure 4.5B reveals that FcγRIIb is expressed at the cell surface only in dbcAMP differentiated cells. The bands marked IgG in Figure 4.5B represent the heavy and light chain of mAb KB61 which

are detected by the secondary anti-mouse:HRP antibody. The increased intensity of these bands in the dbcAMP precipitation is due to the increased expression of FcγRII (and thus binding of KB61) in these cells. In IFN-γ primed cells, although IIAD2 recognised a band of the correct molecular size for FcγRIIb in cell lysates (Figure 4.5A), the immune precipitation experiment performed here failed to demonstrate FcγRIIb at the cell surface as IIAD2 did not recognise a band in KB61 immune precipitates. This means either IIAD2 crossreacts non-specifically with other proteins in cell lysates or that IFN-γ cells express low levels of non-surface expressed FcγRIIb.

4.2.2. FcγRII transiently recruits SHIP following receptor aggregation

In B-cells and Mast cells, FcγRIIb plays a critical negative signalling role. In these cells, FcγRIIb inhibits sustained PLCγ activation and prolonged $InsP_3$ production by recruiting the 5'inositol phosphatase, SHIP (Bolland *et al.*, 1998, Scharenberg *et al.*, 1998) (section 1.2.8.9). Having identified FcγRIIb on the surface of dbcAMP differentiated cells, its role in regulating immune complex mediated signalling in these cells was investigated. In this regard, the potential for FcγRIIb to recruit SHIP was examined.

FcγRII was aggregated over a timecourse using mAb KB61 and goat anti-mouse IgG (Fab specific) as described in section 2.3.1.1. Following aggregation, cell lysates were prepared and FcγRII precipitated with immobilized goat anti-mouse IgG (Fc specific) (section 2.3.4.2.). By using crosslinking antibodies (Fab specific) and precipitating antibodies (Fc specific) with different specificities for mouse IgG, steric problems can be avoided. Immune precipitations were subjected to Western blotting with anti-phosphotyrosine mAbs (clone 4G10) and anti-SHIP polyclonal antibodies (Figure 4.6A). Alternatively, immune precipitates were subjected to *in vitro* inositol phosphatase assays (section 2.4.2) (Figure 4.6B). Following receptor aggregation, a number of tyrosine phosphorylated proteins appear in the FcγRII immune precipitates including a 145kDa protein (Figure 4.6A-top panel). Probing a parallel blot with anti-SHIP polyclonal antibodies reveals the transient association of p145 SHIP with the receptor, appearing within 30 seconds after receptor aggregation (Figure 4.6A-bottom panel). SHIP immunoreactivity is no longer detectable 15 minutes after receptor aggregation. The

presence of SHIP in FcγRII precipitates is confirmed by a transient increase in inositol phosphatase activity associated with the receptor. This activity followed a similar time course to the appearance of SHIP on Western blots (Figure 4.6B). SHIP could not be detected, either by Western blot, or by inositol phosphatase assay in FcγRII immune precipitates following receptor aggregation in IFN-γ treated cells (data not shown). As IFN-γ treated cells express no cell surface FcγRIIb (Figure 4.5B) this data implies that SHIP is recruited by FcγRIIb.

4.2.3. FcγRII aggregation results in the transient association of SHIP with RasGAP and Shc

As well as inhibiting PLCγ and calcium influx, FcγRIIb has been reported to block activation of Ras by recruiting RasGAP to SHIP via the adapter protein p62 Dok (Tamir *et al.*, 2000). To determine if such a mechanism exists in dbcAMP differentiated U937 cells, SHIP was immune precipitated following a timecourse of FcγRII aggregation. Following SDS-PAGE, these immune precipitates were subjected to Western blotting and probed for the presence of RasGAP. Figure 4.7 demonstrates that after aggregation of FcγRII, RasGAP appears in the SHIP immune precipitates, suggesting that RasGAP is recruited to SHIP. The adapter protein, Shc, was also seen to transiently associate with SHIP in response to FcγRII aggregation (Figure 4.7). This SHIP-Shc interaction has recently been implicated in mediating the correct phosphorylation of Shc (Ingham *et al.*, 1999).

As SHIP cannot concomitantly bind to Shc and FcγRIIb (Tridandapani *et al.*, 1999), one possibility was that expression of FcγRIIb might interfere with the correct phosphorylation of Shc and, thus, disrupt Shc dependent signalling. To test this, Shc was immune precipitated following FcγRII aggregation from IFN-γ and dbcAMP treated U937 cells and immune precipitates western blotted for the presence of associated phosphotyrosine containing proteins. Figure 4.8 demonstrates that the Shc complexes formed in response to FcγRII aggregation appear identical regardless of the expression of FcγRIIb. The composition and role of these Shc complexes is discussed in more detail in Chapter 5.

4.2.4. dbcAMP differentiated U937 cells show enhanced FcγRII responses when compared to IFN-γ primed cells

Differentiation with dbcAMP upregulates cell surface expression of both FcγRIIa and FcγRIIb. Conversely, IFN-γ induces little or no change in FcγRII expression and in these cells only FcγRIIa is expressed at the cell surface. To test whether this upregulation of FcγRII expression was accompanied by enhanced signalling, a number of key signalling events activated by FcγRII were compared between IFN-γ and dbcAMP treated U937 cells.

FcγRII was aggregated for 2 minutes with mAb KB61, under identical conditions on both IFN-γ and dbcAMP differentiated cells. Analysis of cell lysates by Western blot with anti-phosphotyrosine antibodies (section 2.3.6.1) demonstrates that dbcAMP differentiated cells show substantially enhanced phosphotyrosine responses when compared to IFN-γ primed cells (Figure 4.9A). As SHIP was found to transiently associate with RasGAP following FcγRII aggregation, FcγRII induced Ras activation was also examined in both IFN-γ and dbcAMP differentiated cells. Figure 4.9B clearly shows that dbcAMP cells show enhanced FcγRII mediated Ras activation. As PKB has been reported as a target for FcγRIIb mediated inhibition (Aman *et al.*, 1998, Jacob *et al.*, 1999) , the ability of FcγRII to couple to PKB was also examined. Activated PKB can be detected in cell lysates on Western blots using antibodies which recognise PKB only when it is phosphorylated on Ser473 (section 2.3.6.3.). Cell lysates were prepared as described above for the phosphotyrosine analysis. Intriguingly, levels of phospho-PKB (Ser473) following FcγRII aggregation were found to be higher in IFN-γ than dbcAMP differentiated cells (Figure 4.9C). No phospho-PKB immunoreactivity could be detected in resting cells.

Previous work from the laboratory has also demonstrated that, following differentiation with dbcAMP, aggregation of FcγRII with mAbs induces a higher proportion of cells to exhibit calcium oscillations than in cells primed with IFN-γ ((Melendez *et al.*, 1998a). Taken together, these data demonstrate that multiple aspects of FcγRII signalling are

enhanced by differentiation with dbcAMP, likely reflecting the increased expression of FcγRII on these cells. Interestingly, the Ras responses are enhanced despite expression of FcγRIIb and the apparent involvement of the Ras inhibitory enzyme, RasGAP. Only PKB was found to be decreased in correlation with FcγRIIb expression, identifying this pathway as a possible target for a SHIP mediated inhibitory signal. These data imply that induced FcγRIIb expression does not abrogate FcγRIIa induced signal transduction but rather modulates the signal generated.

4.2.5. FcγRIIb modulation of FcγRIIa signalling

To determine the role of FcγRIIb in dbcAMP treated cells, a system was developed to differentially aggregate FcγRII subtypes with mAbs. Here, Fab fragments of mAb IV3, along with $F(ab')_2$ fragments of goat anti-mouse IgG were used to specifically aggregate FcγRIIa. Alternatively, mAb KB61 was used with with Fab^2 fragments of goat anti-mouse IgG to co-aggregate FcγRIIa and FcγRIIb. By using $F(ab')_2$ fragments, non-specific recruitment of Fc receptors through Fc interactions with crosslinking antibodies can be avoided.

Using this system of differential aggregation, the ability of FcγRIIa alone or FcγRIIa and FcγRIIb together to activate Ras was assessed. Receptors were aggregated for either 1 minute or 3 minutes and Ras activity measured as described in section 2.3.7. As well as assaying Ras activity, the levels of tyrosine phosphorylation induced in cell lysates was determined by Western blot (section 2.3.6.1) to control for differences in the efficiency of each mAb to aggregate receptors. Figure 4.10 indicates that both IV3 and KB61 induce identical levels of Ras activation following receptor aggregation at both 1 minute and 3 minute timepoints (Figure 4.10 - top panel). A comparison of the tyrosine phosphorylation levels induced by each antibody however indicates that KB61 induces a substantially higher level of tyrosine phosphorylation than IV3 (Figure 4.10 - bottom panel).

Protein kinase B, another target for FcγRIIb mediated inhibitory signalling was also assayed following differential crosslinking (Figure 4.11). FcγRIIa was aggregated alone

(using mAb IV3) or with FcγRIIb (using mAb KB61) over a timecourse and cell lysates prepared. Activated PKB can be detected in cell lysates using antibodies which recognise phospho Ser473-PKB (phospho-PKB) (section 2.3.6.3.). Here, slightly higher levels of phospho-PKB were detectable when FcγRIIa was aggregated alone with mAb IV3 (Figure 4.11 - top panel). Once more, comparison of the levels of tyrosine phosphorylation induced by mAb IV3 aggregation were significantly lower than those induced by KB61 (Figure 4.11 - bottom panel). Thus, as for Ras activation, aggregation of FcγRIIa with mAb IV3 induces comparable levels of phospho-PKB to mAb KB61, despite inducing significantly lower levels of phosphotyrosine.

4.3. Discussion

4.3.1. Expression of FcγRIIb and SHIP recruitment in dbcAMP differentiated U937 cells

The data presented here show that differentiation of the human monocyte cell line, U937, with dbcAMP induces the expression of the inhibitory low affinity IgG receptor FcγRIIb. RT-PCR analysis indicates that the FcγRIIb splice variant expressed is FcγRIIb1 (see section 1.1.3.2). Hybridisation with specific oligonucleotide probes and sequence analysis further confirmed the identity of this FcγRIIb PCR product. To assess whether upregulation of the mRNA for FcγRIIb was reflected at the protein level, Western blot analysis with the FcγRIIb specific mAb, IIAD2 was employed (Weinrich *et al.*, 1996). This confirmed that FcγRIIb was surface expressed solely in dbcAMP treated U937 cells and further that expression levels were comparable to those seen in the EDR B-cell line. Taken together, these data also rule out the possibility that this induced expression might result from crossreactivity with the third FcγRII gene FcγRIIc (see section 1.1.3.2). Concomitant with this induction of FcγRIIb, FcγRIIa was also upregulated in dbcAMP differentiated cells as assessed by RT-PCR, Northern blot and FACS analysis.

The role of FcγRIIb1 has been extensively studied in B-cells and mast cells (section 1.2.8). Here, co-ligation of FcγRIIb with the BCR in B-cells or FcεRI in mast cells results

in inhibition of prolonged PLC activation, $InsP_3$ production and calcium influx (Ono *et al.*, 1996, Bolland *et al.*, 1998, Scharenberg & Kinet, 1996). The finding that FcγRIIb expression was induced in dbcAMP differentiated U937 cells was entirely unexpected as previous work from our laboratory has demonstrated that in these cells, immune complex stimulation results in activation of PLCγ1 and prolonged calcium transients (Davis *et al.*, 1995a, Melendez *et al.*, 1999b, Melendez *et al.*, 1998a). Initially, therefore studies were undertaken to assess whether this FcγRIIb expressed in dbcAMP differentiated cells was capable of recruiting the inhibitory apparatus so well characterised in B-cells and mast cells.

In murine B-cells and mast cells, the inhibitory properties of FcγRIIb centre round the recruitment of the 5'inositol phosphatase, SHIP, to the phosphorylated ITIM within the receptors cytoplasmic tail (Ono *et al.*, 1996, Fong *et al.*, 1996, Dambrosio *et al.*, 1996a). Similarly, I demonstrate that SHIP transiently associates with FcγRII, as assessed by both Western blot and associated 5'inositol phosphatase activity. Conversely, in U937 cells primed with IFN-γ (which only express FcγRIIa) no SHIP or 5' inositol phosphatase activity could be detected in FcγRII precipitates. Taken together these data support FcγRIIb mediated recruitment of SHIP in dbcAMP U937 cells.

It is important to point out that previous work from our laboratory (Chapter 3) and others has suggested that the SHIP pathway might be activated by FcγRs in the absence of an ITIM bearing receptor. Thus FcγRI and FcγRII aggregation induce the tyrosine phosphorylation of SHIP and its association with the adapter protein, Shc, in IFN-γ primed U937 cells (Cameron & Allen, 1999), untreated U937 and THP-1 cells (Maresco *et al.*, 1999). Recent data from B-lymphocytes however demonstrates that BCR induced interactions between SHIP and Shc, rather than being inhibitory, play a crucial role in directing the phosphorylation of Shc (Ingham *et al.*, 1999). Using SHIP deficient DT40 chicken B-cells, Ingham *et al* demonstrate that, in the absence of SHIP, the BCR fails to induce significant Shc phosphorylation. In the light of this data, it seems likely that our observed interactions between SHIP and Shc induced by FcγRs in IFN-γ primed U937 cells are independent of the inhibitory actions of SHIP and are more likely to be important in directing the correct phosphorylation of Shc (see Chapter 3).

In dbcAMP differentiated U937 cells, FcγRIIb appears to recruit SHIP to the immune complex. I hypothesised that this recruitment might interfere with SHIP mediated Shc phosphorylation, consistent with previous reports that SHIP cannot concomitantly bind to FcγRIIb and Shc (Tridandapani *et al.*, 1999). To test this theory, Shc complexes induced by FcγRIIa alone in IFN-γ treated cells and those induced by co-aggregation of FcγRIIa and FcγRIIb in dbcAMP differentiated cells were compared. Following FcγRII aggregation in either IFN-γ or dbcAMP treated cells, Shc immune precipitates were probed for the presence of tyrosine phosphorylated proteins, and also for SHIP. The equal levels of SHIP detected in association with Shc, and the near identical pattern of tyrosine phosphorylated proteins associated with Shc (discussed further in Chapter 5) under these different conditions of differentiation suggests that this is not the case. This data implies two distinct roles for SHIP in these cells.

In summary, differentiation with dbcAMP induces the expression of FcγRIIb, which when co-aggregated with FcγRIIa, transiently recruits SHIP. This recruitment of SHIP is specific to dbcAMP cells and appears independent of the previously reported, ITIM independent, SHIP-Shc interactions induced by Fcγ receptors (Cameron & Allen, 1999).

4.3.2. FcγRIIb modulation of immune complex mediated signalling in dbcAMP differentiated U937 cells

Differentiation of U937 cells with dbcAMP both upregulates the ITAM bearing FcγRIIa and induces expression of the ITIM bearing FcγRIIb. Further, FcγRI expression in these dbcAMP differentiated cells is downregulated (Chapter 6 - Figure 6.5) and FcγRIII expression is not detectable (data not shown). Thus, the outcome of this differentiation is a U937 cell in which immune complex induced signalling is mediated primarily by FcγRII isoforms. Use of mAbs to aggregate specifically FcγRII in either dbcAMP differentiated cells or IFN-γ primed cells indicates that upregulation of FcγRII expression by dbcAMP is accompanied by enhanced coupling of FcγRII to tyrosine phosphorylation events and Ras activation. In contrast, coupling of FcγRII to PKB activation was found to be more robust in IFN-γ primed cells than in dbcAMP differentiated cells..

DbcAMP differentiation of U937 cells results in upregulation of FcγRIIa and the appearance of FcγRIIb at the cell surface. The enhanced responses to FcγRII aggregation in these cells is likely to result from enhanced FcγRIIa expression. However, in other cell systems FcγRIIb has been shown to act as an inhibitory receptor. Therefore, the data presented here on Ras activation,together with the previous data on PLCγ1 activation and calcium transients (Davis *et al.*, 1995a, Melendez *et al.*, 1998a), raise questions concerning the role of FcγRIIb in the modulation of immune complex mediated signalling pathways in U937 cells. One possibility is that FcγRIIb is required to set a threshold or ceiling for FcγRIIa signalling. Such a mechanism may not be required at lower levels of FcγRIIa expression, or under circumstances where other Fcγ receptors play a dominant role in immune complex signalling, such as FcγRI in IFN-γ treated cells. Another possibility is that FcγRIIb, when co-expressed with FcγRIIa, does not play an inhibitory role, but rather forms part of an activating complex. As no data exist for the effect of FcγRIIb on FcγRIIa signalling, attempts were made to develop a system to differentially aggregate FcγRIIa and FcγRIIb with mAbs.

In murine B-cells, a number of pathways have been identified as targets for FcγRIIb mediated inhibition. These include the PLCγ/$InsP_3$/calcium pathway, the Ras/MAP kinase pathway and PKB (section 1.2.8). Two approaches were taken to assess the effects of FcγRIIb expression on signalling.

Firstly, the signalling profiles activated by FcγRII aggregation in IFN-γ primed cells and dbcAMP differentiated cells were compared. Here, IFN-γ primed cells express solely FcγRIIa at the cell surface, while dbcAMP differentiated cells express both FcγRIIa and FcγRIIb. Data here indicate that both tyrosine phosphorylation and Ras activation are enhanced in dbcAMP differentiated cells. As FcγRIIa is upregulated in dbcAMP differentiated cells, these enhanced responses are likely a result of increased expression of the activating receptor. PKB, conversely, was found to be phosphorylated more robustly following FcγRII aggregation in IFN-γ treated cells than in the dbcAMP differentiated cells. This identifies PKB, which is inhibited by FcγRIIb in B-cells (Jacob *et al.*, 1999, Aman *et al.*, 1998), as a potential target for FcγRIIb mediated inhibition in U937 cells.

Secondly, to avoid complications associated with differentiation, mAbs were used in an effort to differentially aggregate FcγRIIa and FcγRIIb within the same, dbcAMP differentiated cells. Here, using a mAb which recognises only FcγRIIa (IV3) or a mAb which recognises all FcγRII isoforms (KB61) attempts were made to assess the effects of FcγRIIb co-aggregation on Ras and PKB activation. The experimental conditions used were designed to maximally aggregate surface FcγRII. Clearly, this is a non-physiological stimulus as, *in vivo*, immune complexes are variable both in size and Ig composition. However, the wide variety of receptors expressed on myeloid cells, with their varying affinities for different IgG subclasses (IgG1-4) and Ig classes (ie. IgA and IgE) results in highly variable ratios of receptor recruitment to any given immune complex. It is therefore necessary to use mAbs to trigger activation in a precise manner, to begin to understand the relative contributions of specific receptors.

Data generated using this system of differential crosslinking has indicated some differences in the responses generated by the FcγRIIa specific mAb, IV3, and the pan-anti-FcγRII mAb KB61. Aggregation of FcγRII with either mAb results in equal levels of both PKB and Ras activation. However, KB61 was found to couple to consistently higher phosphotyrosine responses. Thus, the relative activation of Ras or PKB responses to phosphotyrosine responses is lower when aggregating both receptors together with mAb KB61. This is consistent with FcγRIIb playing a role tempering FcγRIIa responses. These data might also imply that co-aggregation of FcγRIIa with FcγRIIb results in enhanced coupling to tyrosine kinase activation.

Intriguingly, though FcγRIIa and FcγRIIb show more than 95% homology in their extracellular domains, their affinities for different IgG subtypes differ (section 1.1.3.4) (van de Winkel & Capel, 1993). This raises the possibility that the cellular response to immune complexes will depend dramatically on the proportions of different IgG subtypes within any given immune complex. For example, immune complexes with high levels of IgG2 might be expected to preferentially aggregate FcγRIIa whereas IgG4 complexes will aggregate a higher proportion of FcγRIIb. Such a system would allow the cell to integrate its response according to the composition of a given immune complex, and thus react

appropriately. As different IgG isotypes are associated with different types of immune response, such a system could have broad implications in terms of immune regulation.

As well as modulating Fcγ receptor signal transduction, a number of other potential roles for FcγRIIb exist. It is quite possible, for example, that FcγRIIb might exert its inhibitory actions on other Fc receptors such as FcεRI or FcαRI, which are also present in U937 cells. Further, FcγRII has been shown to bind C-reactive protein (CRP) providing another potential target for modulation (Bharadwaj *et al.*, 1999). In addition to modulating signal transduction, FcγRIIb might play a role in the targeting of immune complexes to specific subcellular compartments. In B-cells, for example, FcγRIIb1 has been reported to block BCR bound antigen presentation by inhibiting endocytosis (Amigorena *et al.*, 1992a, Minskoff, Matter & Mellman, 1998). A potential role for U937 expressed FcγRIIb in the regulation of immune complex bound antigen presentation has not been ruled out.

In summary, the modulating effects of FcγRIIb on macrophage signal transduction appears distinct from its role in B-cells, reflecting the differing physiological roles that FcγRs play in these different cell types. Thus, in B-cells, FcγRIIb acts as an inhibitory feedback mechanism to switch B-cells 'off'. In myeloid cells the role of FcγRIIb appears more flexible, with the nature of the signal transduced likely to depend more on the ratio of activating and inhibitory receptors aggregated. The overall upregulation of FcγRII in these cells is likely to reflect an enhanced role for dbcAMP differentiated macrophage like cells in the clearance of immune complexes. This is supported by the fact that FcγRII (as opposed to FcγRI) is a low affinity receptor which only recognises pre-formed immune complexes. By expressing different isoforms of FcγRII with differing affinities for IgG subtypes and differing signalling motifs within their cytoplasmic tails, cells will be able to respond according to the composition of the specific immune complexes they encounter. In conclusion, the data presented here indicate a novel role for human FcγRIIb in the modulation of macrophage activation.

Figure 4.1 Northern blot analysis of FcγRII expression.

U937 cells were treated with either IFN-γ or dbcAMP and harvested at the times shown (hours). Total RNA (20 μg/sample) was subjected to Northern blot analysis. Blots were hybridised with ^{32}P-labeled cDNA probe specific for FcγRII. Bands were visualised by autoradiography (top panel). The bottom panel shows equal loading of total RNA as assessed by ethidium bromide staining of the RNA gel prior to blotting. The bands indicated are the 18S and 28S ribosomal subunits as indicated.

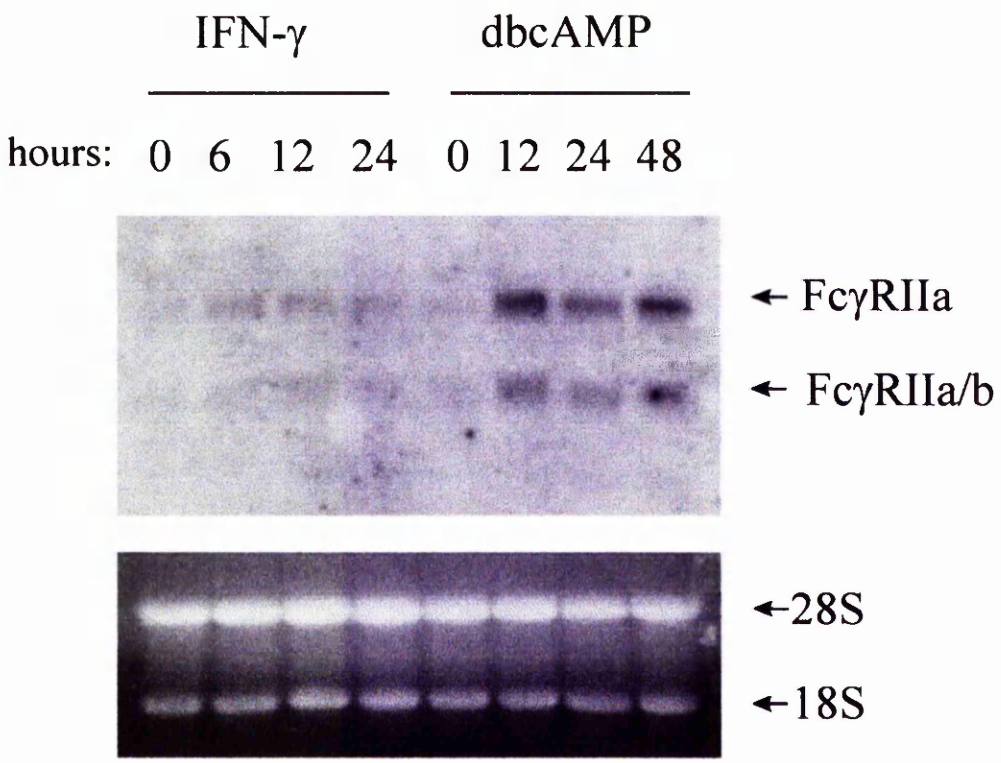

IFN-γ
dbcAMP
hours: 0 6 12 24 0 12 24 48
← FcγRIIa
← FcγRIIa/b
←28S
←18S

Figure 4.2 Plan for RT-PCR amplification of FcγRIIa and FcγRIIb cDNAs.

A single forward primer crossreactive for both FcγRIIa and FcγRIIb (FOR) was designed in conjunction with specific reverse primers for either FcγRIIa (REV2A) or FcγRIIb (REVB). The FcγRIIa primers predict a PCR product of 719 bp. For FcγRIIb, the primers flank a splice site in the FcγRIIb sequence (marked ↓) such that FcγRIIb1 would give rise to a 289 bp fragment while FcγRIIb2 would give rise to a 232 bp fragment.

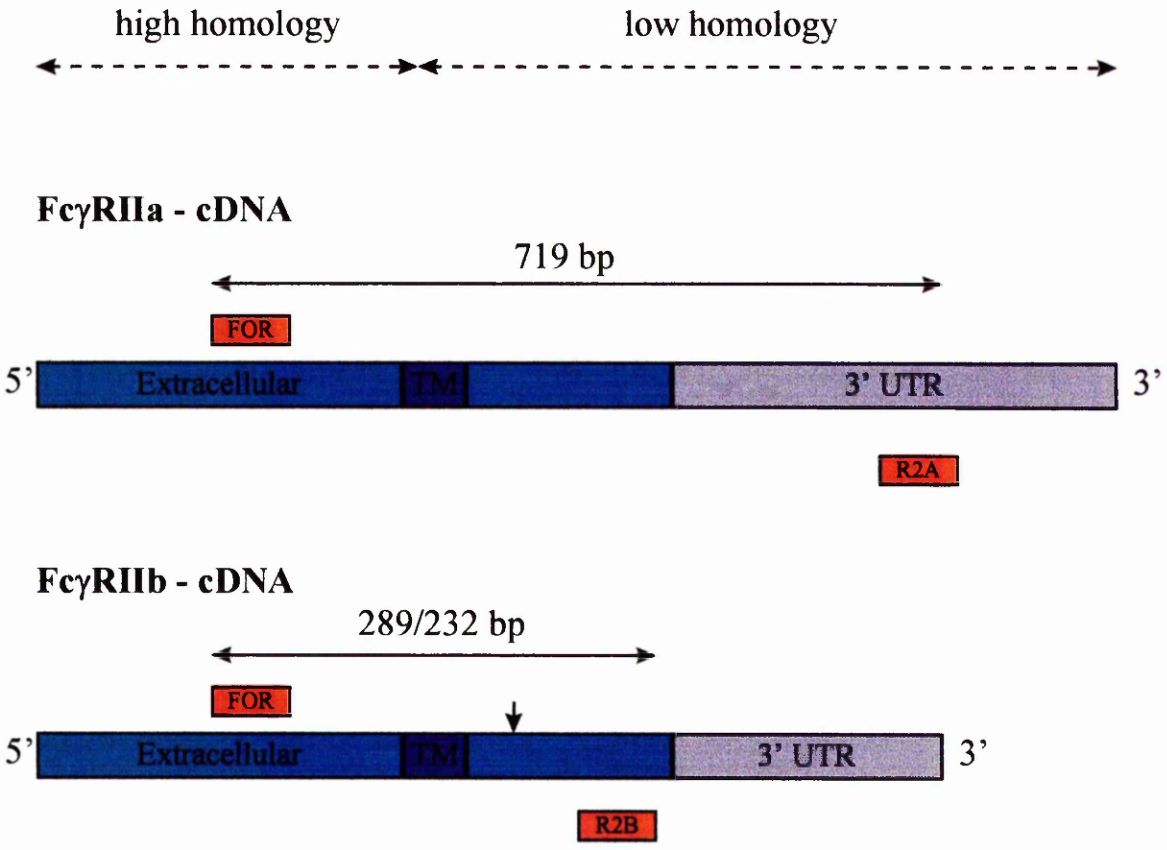

high homology
low homology
FcγRIIa - cDNA
719 bp
FOR
5'
Extracellular
TM
3' UTR
3'
R2A
FcγRIIb - cDNA
289/232 bp
FOR
5'
Extracellular
TM
3' UTR
3'
R2B

Figure 4.3 Semi quantitative RT-PCR analysis of FcγRIIa and FcγRIIb expression.

U937 cells were treated with either IFN-γ or dbcAMP and harvested at the times shown (hours). Following preparation of cDNA by reverse transcription, PCR was used to amplify either FcγRIIa and FcγRIIb or cyclophilin as described in the materials and methods (section 2.4.8). PCR products were visualised by Southern blot hybridisation with a crossreactive cDNA probe for FcγRII (top panel). Cyclophilin was used as an internal standard to ensure equal loading of cDNA, as indicated. Bands were visualised by autoradiography.

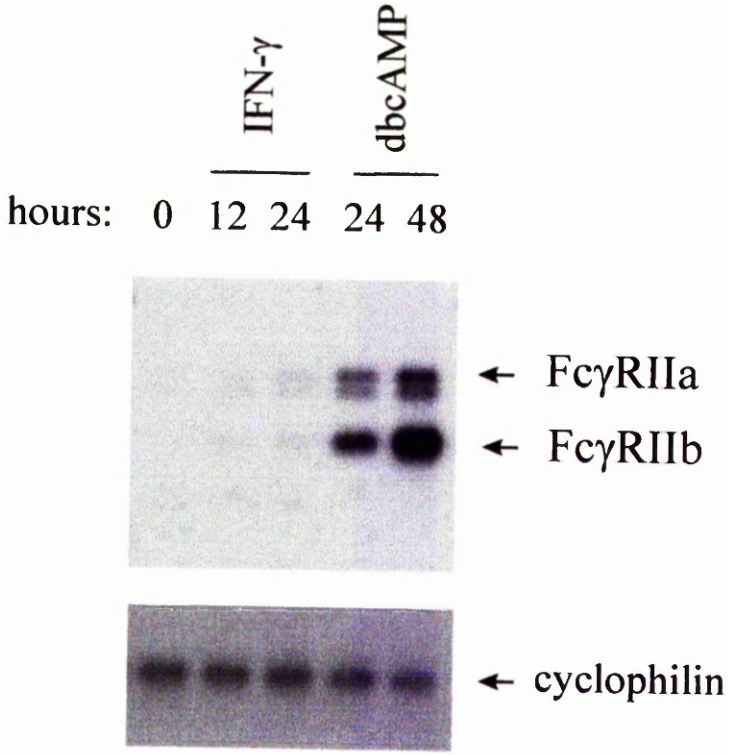
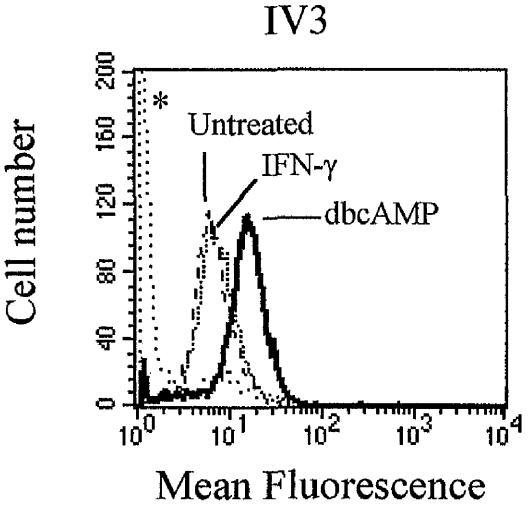

IFN-γ
dbcAMP
hours: 0 12 24 24 48
FcγRIIa
FcγRIIb
cyclophilin

Figure 4.4 FACS analysis of FcγRIIa and total FcγRII surface expression.

Untreated U937 cells and cells treated with either IFN-γ (24 hours) or dbcAMP (48 hours) were labeled with either the FcγRIIa specific mAb IV3 (top panel), or total FcγRII specific mAb KB61 (bottom panel). Human IgG (3μM) was also added to prevent non-specific binding of antibodies to Fc receptors. A goat anti-mouse IgG:FITC was used as a secondary antibody and cells were subjected to FACS analysis. Control cells with no primary antibody were also included (indicated by *).

A


IV3
Cell number
Mean Fluorescence
Untreated
IFN-γ
dbcAMP
*
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4

B

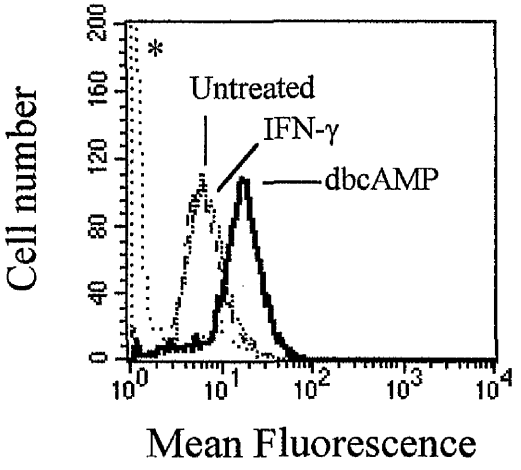
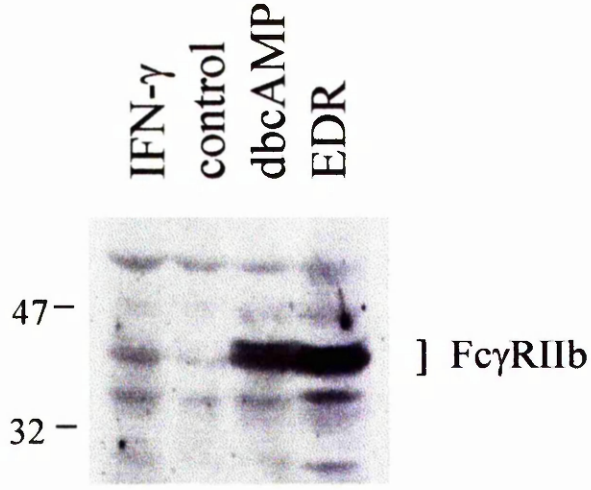

KB61
Cell number
Mean Fluorescence
Untreated
IFN-γ
dbcAMP
*
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4

Figure 4.5 Western blot analysis of FcγRIIb expression.

(A) Cell lysates (20 μg/sample) from untreated U937 cells (control) and cells treated with either IFN-γ (24 hours) or dbcAMP (48 hours) were subjected SDS-PAGE and Western blot analysis with the FcγRIIb specific mAb IIAD2. Cell lysate (20 μg) from the EDR human B-cell line was also included as a positive control. An anti-mouse:HRP was used as a secondary antibody and bands were visualised using the ECL system (Amersham).

(B) Surface expressed FcγRII was immune precipitated using the pan-FcγRII mAb KB61 as described in the results section. Immune precipitates were subjected to Western blot analysis with the FcγRIIb specific mAb IIAD2 as described for (A). The heavy and light chains of the precipitating antibody KB61, which are detected by the secondary HRP conjugate, are indicate by IgG.

A


IFN-γ
control
dbcAMP
EDR
47
32
] FcγRIIb

B

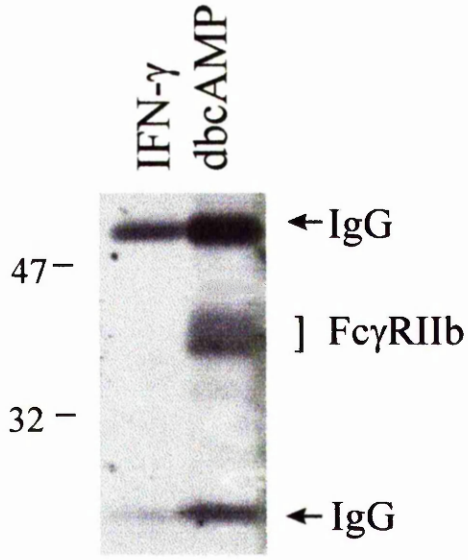
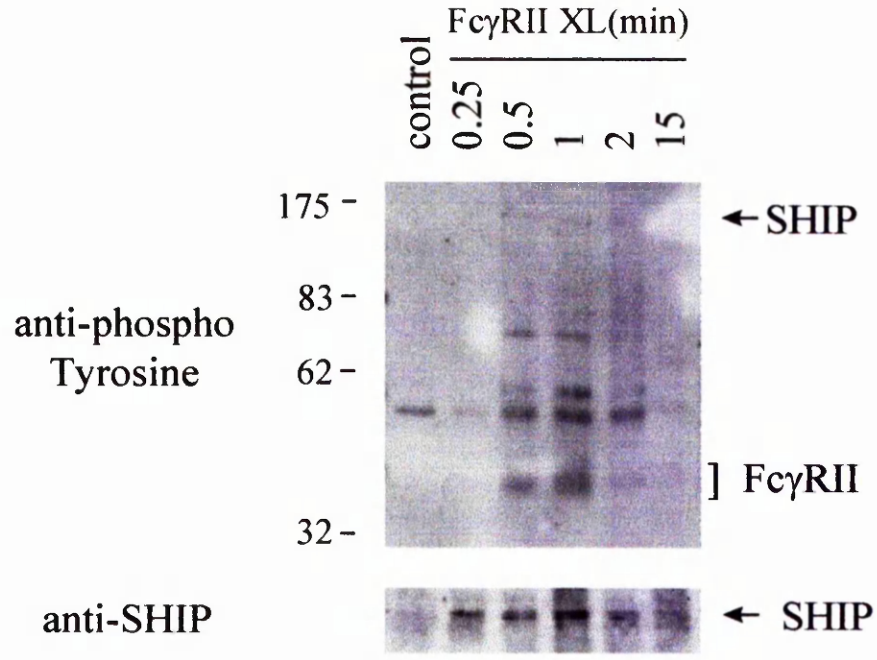

IFN-γ
dbcAMP
47
32
←IgG
] FcγRIIb
←IgG

Figure 4.6. Western blot analysis of FcγRII immune precipitates.
Cells were treated with dbcAMP for 48 hours prior to the experiment. FcγRII was aggregated for the times indicated. Cells where no crosslinking antibody was added were included as a control. FcγRII was precipitated via KB61 using goat anti-mouse (Fc specific) IgG conjugated agarose. Immune precipitates were subjected either to Western blot analysis (A) or in vitro inositol phosphatase assays (B). For Western blot analysis (A), membranes were probed with either anti-phosphotyrosine mAb 4G10 (top panel) or goat polyclonal anti-SHIP antibodies (bottom panel). For inositol phosphatase assays (B) data are the mean ± standard deviation (error bars) of triplicate measurements and are representative of three separate experiments.

A


FcγRII XL(min)
control
0.25
0.5
1
2
15
175
83
62
32
anti-phospho Tyrosine
← SHIP
] FcγRII
anti-SHIP
← SHIP

B

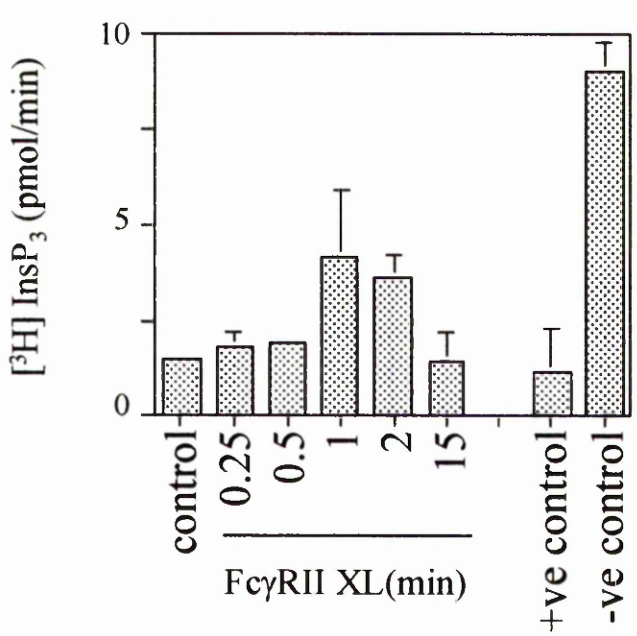

10
5
0
[3H] InsP3 (pmol/min)
control
0.25
0.5
1
2
15
FcγRII XL(min)
+ve control
-ve control

Figure 4.7 SHIP interacts with RasGAP and Shc following FcγRII aggregation.

Cells were treated with dbcAMP for 48 hours prior to the experiment. FcγRII was aggregated using mAb KB61 and goat anti-mouse IgG for the times indicated (min). Cells where no crosslinking antibody was added were included as a control. Following preparation of cell lysates, SHIP was immune precipitated with polyclonal goat anti-SHIP antibodies. Immune precipitates were subjected to Western blot analysis with anti-RasGAP mAbs (top panel), anti-Shc mAbs (middle panel) and goat anti-SHIP polyclonal antibodies (bottom panel).

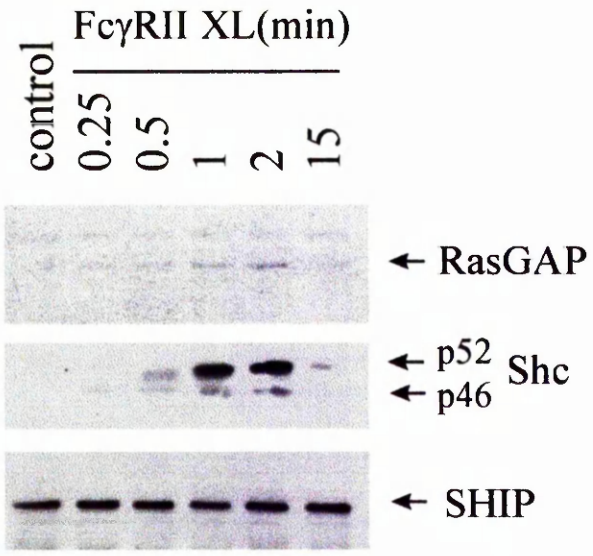

FcγRII XL(min)
control
0.25
0.5
1
2
15
RasGAP
p52
p46
Shc
SHIP

Figure 4.8 FcγRIIb expression does not effect the formation of Shc complexes.

U937 cells were treated with IFN-γ or dbcAMP as indicated. FcγRII was aggregated for 2 minutes using mAb KB61 and goat anti-mouse IgG. Control cells were incubated with goat anti-mouse IgG alone. Shc was immune precipitated with rabbit polyclonal anti-Shc antibodies as previously described. Immune precipitates were analysed by Western blot with anti-phosphotyrosine mAb 4G10 (top panel), goat polyclonal anti-SHIP antibodies (middle panel) or anti-Shc mAb (bottom panel).

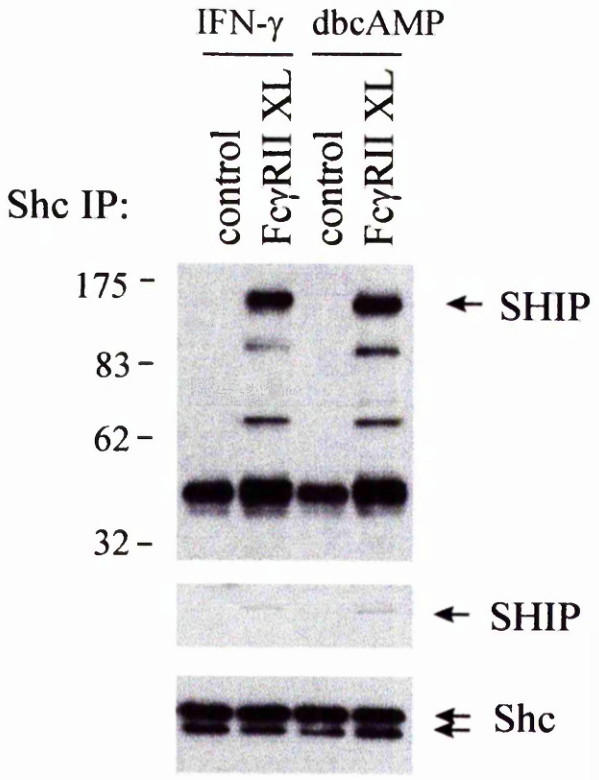
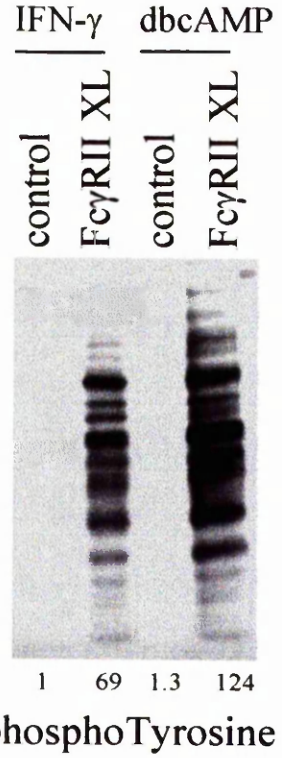

IFN-γ
dbcAMP
control
FcγRII XL
control
FcγRII XL
Shc IP:
175 -
83 -
62 -
32 -
SHIP
SHIP
Shc

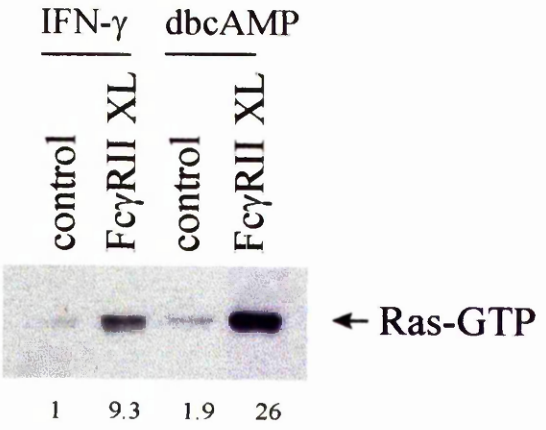
Figure 4.9 dbcAMP differentiated cells show enhanced FcγRII induced signalling.

U937 cells were treated with dbcAMP for 48 hours prior to experiments. For (A) and (B) FcγRII was aggregated for 2 minutes using mAb KB61 and goat anti-mouse IgG. For (C) FcγRII was aggregated for 1 minute or 3 minutes as indicated. Control cells were incubated with goat anti-mouse IgG alone. (A) Cell lysates (20 μg/sample) were subjected to Western blot analysis with anti-phosphotyrosine mAb 4G10. Numbers beneath blot indicate the relative intensity of phosphotyrosine in each lane as assessed by densitometry, with control arbitrarily set as 1. (B) Ras activity was assessed in cell lysates as described in section 2.3.7. Numbers beneath blot indicate the relative intensities of the Ras band as assessed by densitometry, with control arbitrarily set as 1. (C) PKB-Ser473 phosphorylation in cell lysates (20μg/sample) was assessed by Western blot using polyclonal anti-phospho Ser473-PKB antibodies (top panel). Blots were reprobed with anti-PKB antibodies (bottom panel) to ensure comparable loading of cell lysates. Numbers beneath blot indicate the relative intensity of bands as assessed by densitometry, with control arbitrarily set as 1. These data are corrected for equal loading.

A


IFN-γ
dbcAMP
control
FcγRII XL
control
FcγRII XL
1
69
1.3
124
phosphoTyrosine

B


IFN-γ
dbcAMP
control
FcγRII XL
control
FcγRII XL
← Ras-GTP
1
9.3
1.9
26

C

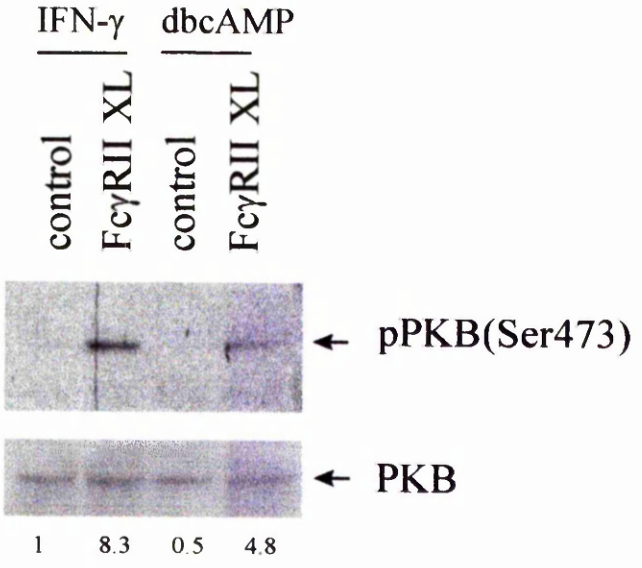

IFN-γ
dbcAMP
control
FcγRII XL
control
FcγRII XL
← pPKB(Ser473)
← PKB
1
8.3
0.5
4.8

Figure 4.10 Activation of Ras by FcγRIIa aggregation or FcγRIIa/FcγRIIb co-aggregation.

U937 cells were differentiated with dbcAMP for 48 hours. FcγRIIa was aggregated alone with mAb IV3 (as indicated) and goat anti-mouse IgG for 1 minute or 3 minutes. Alternatively, FcγRIIa/FcγRIIb were co-aggregated with mAb KB61 (as indicated) and goat anti-mouse IgG for I minute or 3 minutes. Control cells were incubated with goat anti-mouse IgG alone. Ras activity was assessed in cell lysates (top panel) as described in section 2.3.7. Tyrosine phosphorylation of proteins in cell lysates was assessed by Western blotting with anti-phosphotyrosine mAb 4G10. Numbers beneath blots indicate the relative intensity of bands as assessed by densitometry with control arbitrarily set as 1. Data is normalised for equal loading.

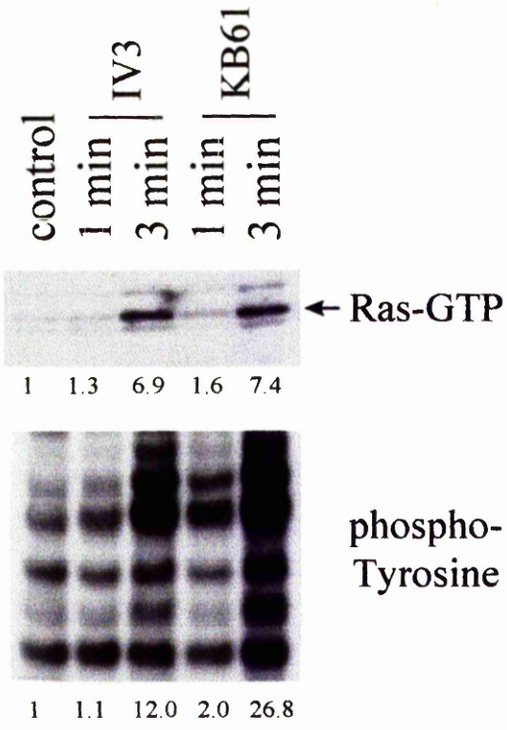
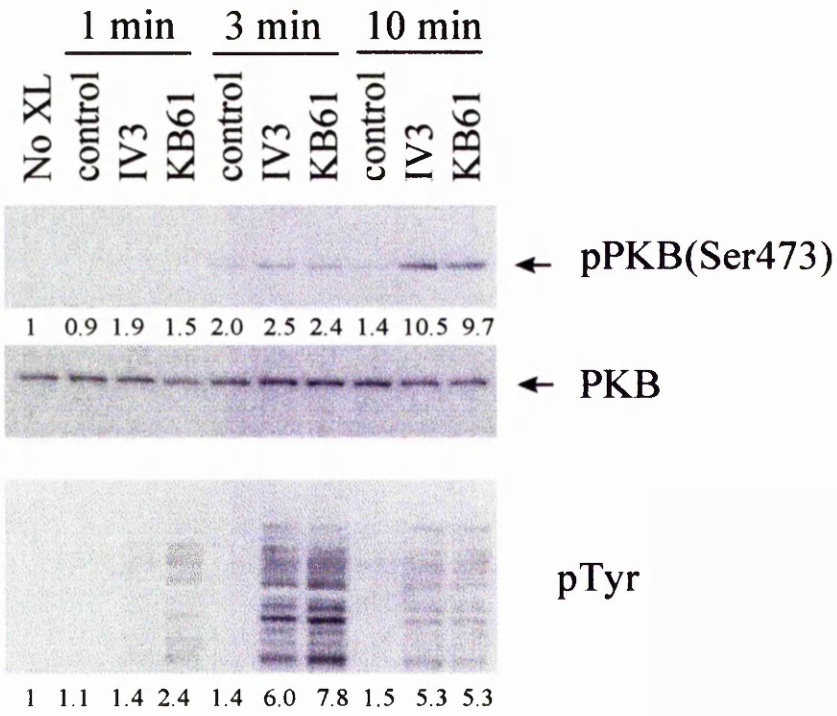

control
1 min
3 min
1 min
3 min
IV3
KB61
Ras-GTP
1 1.3 6.9 1.6 7.4
phospho-
Tyrosine
1 1.1 12.0 2.0 26.8

Figure 4.11 Activation of PKB by FcγRIIa aggregation or FcγRIIa/FcγRIIb co-aggregation.

U937 cells were differentiated with dbcAMP for 48 hours. FcγRIIa was aggregated alone with mAb IV3 (as indicated) and goat anti-mouse IgG for the times indicated. Alternatively, FcγRIIa/FcγRIIb were co-aggregated with mAb KB61 (as indicated) and goat anti-mouse IgG. Control cells were incubated with goat anti-mouse IgG alone. PKB phosphorylation in cell lysates (20μg/sample) was assessed by Western blot using polyclonal anti-phosphoPKB (Ser473) antibodies (top panel) as described in section 2.3.6.3. Blots were reprobed with anti-PKB antibodies (middle panel) to ensure comparable loading of cell lysates. Tyrosine phosphorylation of proteins in cell lysates was assessed by Western blotting with anti-phosphotyrosine mAb 4G10. Numbers beneath blots indicate the relative intensity of bands as assessed by densitometry, with control arbitrarily set as 1. Data is normalised for equal loading.


1 min
3 min
10 min
No XL
control
IV3
KB61
control
IV3
KB61
control
IV3
KB61
pPKB(Ser473)
1 0.9 1.9 1.5 2.0 2.5 2.4 1.4 10.5 9.7
PKB
pTyr
1 1.1 1.4 2.4 1.4 6.0 7.8 1.5 5.3 5.3

Chapter 5

Differential coupling of FcγRI and FcγRII to adapter proteins, PLCγ1, small G-proteins and the MAP kinase cascades

5.1. Introduction

Fcγ receptors (FcγR) play a critical role in the coordination of the immune response to antibody:antigen complexes by triggering the production of multiple cytokines, inflammatory mediators and cell surface molecules. The specific response to immune complexes depends on multiple factors including cell type, and differentiation state, and the Fcγ receptor subtypes involved. The signalling events which link FcγR engagement to the nucleus have not been well defined. It is clear, however, that activation of signalling pathways by FcγR as well as other immunoreceptors (including BCR, TCR and FcεRI) involves the activation of Src and Syk family tyrosine kinases (Agarwal *et al.*, 1993, Cambier, 1995). These kinases in turn phosphorylate multiple substrates essential for signal transduction (Liao *et al.*, 1992, Scholl *et al.*, 1992). Recently, progress has been made towards understanding the molecular mechanisms linking tyrosine kinase activation to specific effector pathways.

Among the many substrates of Src and Syk family tyrosine kinases are a number of adapter proteins which have no intrinsic activity. Rather, these adapter proteins contain multiple protein:protein and protein:lipid interaction domains. Studies of several of these adapter proteins have revealed their critical roles in coordinating specific biological responses. It appears that these adapter proteins act as complex molecular scaffolds, linking tyrosine kinase activation with specific effector pathways (Rudd, 1999, Myung *et al.*, 2000).

PLCγ activation plays a central role linking Fc and antigen receptors to the generation of second messengers and the mobilisation of calcium (section 1.2.4) (Berridge, 1993, Tsien & Tsien, 1990, Putney, 1986). Following receptor engagement, PLCγ is recruited to the

plasma membrane where it catalyses the conversion of the membrane lipid, PtdIns(4,5)P_2, into diacylglycerol (DAG) and $InsP_3$. $InsP_3$ in turn induces the release of calcium from intracellular stores, which is followed by store operated calcium influx through plasma membrane calcium channels (Hoth & Penner, 1992). This calcium mobilisation plays a role in the activation of a number of transcription factors (section 1.2.4.1) (Dolmetsch *et al.*, 1998, Hu *et al.*, 1999, Li *et al.*, 1998). Further, the PLCγ product DAG, along with calcium release, plays a key role in the activation of PKC isoforms (Nishizuka, 1992, Dekker & Parker, 1994). Recently, a number of adapter proteins have been identified, which link antigen receptors (the BCR and TCR) and FcεRI to PLCγ activation.

In the cases of the TCR and FcεRI, the adapter proteins, LAT, and SLP-76, are critical for PLCγ activation (Finco *et al.*, 1998, Yablonski *et al.*, 1998, Saitoh *et al.*, 2000, Turner & Kinet, 1999). LAT is an integral membrane protein which contains multiple tyrosine residues within its cytoplasmic tail. Conversely, SLP-76 is a cytoplasmic adapter which bears a number of protein:protein interaction motifs, including an N-terminal acidic domain containing multiple tyrosine residues, a central proline rich region (which interacts with SH3 domains) and a C-terminal SH2 domain for phosphotyrosine binding. Following aggregation LAT, which co-localises with activated receptors in lipid rafts, becomes rapidly tyrosine phosphorylated by Src and Syk family tyrosine kinases. Phosphorylated LAT recruits PLCγ to the plasma membrane, which is necessary, but not sufficient for activation of PLCγ (Zhang *et al.*, 1998). For activity, PLCγ must be phosphorylated, not only by Syk family kinases, but also by Tec family kinases (Itk for the TCR, Btk for FcεRI) (Scharenberg & Kinet, 1998). Thus, the adapter protein SLP-76, another Syk substrate, appears to link LAT and PLCγ with Tec family kinases. SLP-76 can simultaneously interact with both LAT (via the adapter protein, Gads), and Tec family kinases. Further, SLP-76 can directly bind the SH3 domain of PLCγ.

To complicate matters, SLP-76 and LAT, also play a critical role in linking the TCR and FcεRI to the activation of Ras and Rac GTPases (Finco *et al.*, 1998, Bubeck Wardenburg *et al.*, 1998). Here, tyrosine kinase dependent activation of small GTPases appears to be governed by the recruitment of specific guanine nucleotide exchange factors (GEFs) to the plasma membrane. These GEFs promote the exchange of GDP for GTP bound to small

G proteins and, thus, facilitate their activation (section 1.2.5.2). Recruitment of these exchange factors is facilitated by adapter protein scaffolds.

In the case of Ras, activation appears to be governed by recruitment of the Ras GEF, Sos to the plasma membrane (Gale *et al.*, 1993, Li *et al.*, 1993). The critical adapter protein, Grb2, binds to Sos via its two SH3 domains. The single SH2 domain of Grb2 can bind to tyrosine phosphorylated LAT, thereby recruiting Sos to the plasma membrane where activation of Ras takes place (Kane *et al.*, 2000). It should be noted that the adapter proteins involved in the recruitment of Grb2:Sos differ according to receptors and cell types. Thus, in B-cells, which do not express LAT or SLP-76, the adapter protein Shc has been implicated in the recruitment of Grb2-Sos complexes to the BCR (Harmer & DeFranco, 1997).

The activation of Rac appears to follow a similar pattern to Ras. For the TCR and FcεRI, the Rac GEF, Vav, is recruited to the membrane through SH3 domain interactions with the adapter protein, SLP-76. SLP-76 forms a poorly defined adapter protein complex, containing Grb2 or the related adapter, Gads, which binds to the membrane tethered LAT (Liu *et al.*, 1999, Kane *et al.*, 2000, Turner & Kinet, 1999).

Ras, Rac and Rho regulate a wide range of cellular processes from cytoskeletal reorganisation and endocytosis through to modulation of transcription. Many of the transcriptional responses initiated by these proteins are mediated through activation of the MAP kinases (MAPKs) (Genot & Cantrell, 2000, Aspenstrom, 1999, Su & Karin, 1996).

The MAPKs are a growing family of serine-threonine protein kinases which are activated by a wide variety of extracellular stimuli (Garrington & Johnson, 1999, Su & Karin, 1996). The MAPKs can be divided into a number of subgroups, including the extracellular regulated kinases (ERKs), c-Jun N-terminal or stress activated protein kinases (JNK/SAPK) and the p38 group of kinases. Among the substrates of these kinases are a wide variety of transcription factors and, thus, different activation patterns

of the MAPKs (ERK, JNK, p38) are likely to lead to differential expression of genes (section 1.2.6).

ERK, p38 and JNK are activated by a many immunoreceptors, including the BCR, TCR, FcεRI and FcγRs (Hashimoto *et al.*, 1998, Turner & Kinet, 1999, Rose *et al.*, 1997). However, the mechanisms and functional consequences of their activation varies greatly depending on the cell type and the receptors involved. The best understood pathway to MAPK activation is the Ras/Raf/MEK/ERK pathway. Here, activated Ras binds to and activates the serine/threonine kinase Raf-1. Raf-1 then phosphorylates and stimulates MEK which in turn activates ERK. Similar, but less well defined pathways exist for the JNK and p38 families of MAPK (Coso *et al.*, 1995). For example, activated Rac binds to, and activates p21 activated kinase (PAK) which can initiate kinase cascades leading to the activation of p38 and JNK (Zhang *et al.*, 1995, Coso *et al.*, 1995). It is clear, however, that regulation of the MAPK pathways is complex, with multiple distinct signalling events being integrated to give a specific response.

Previous work from our laboratory has demonstrated that FcγRI couples to a novel signalling pathway in IFN-γ primed U937 cells. Here, FcγRI aggregation leads to the tyrosine kinase dependent activation of phospholipase D, sphingosine kinase and subsequent calcium mobilisation (Melendez *et al.*, 1998b). Surprisingly, no PLCγ activity can be detected. Conversely, aggregation of FcγRII in the same cell type leads to a robust activation of PLCγ and $InsP_3$ dependent calcium mobilisation (Melendez *et al.*, 1998a). The mechanism responsible for this fundamental difference in signalling is not clear.

To investigate the mechanism governing PLCγ activation in U937 cells, adapter proteins have been examined, which are known to be involved with PLCγ activation in other cell types. Here, I demonstrate that SLP-76 forms distinct complexes in response to FcγRI and FcγRII activation, providing a potential mechanism for the differential activation of PLCγ by the two receptors. Further, I demonstrate that SLP-76 differentially interacts with the adapters, Grb2 and Shc, which are implicated in the activation of Ras and Rac. Following on from these differences, the coupling of FcγRI and FcγRII to Ras, Rac and the downstream activation of the MAP kinase cascades is addressed.

5.2. Results

5.2.1. Regulation of adapter protein complexes by FcγRI and FcγRII

Immune receptor activation of multiple signalling pathways, including the activation of PLCγ, small GTPases, and MAPKs, involves the formation of adapter protein complexes. These adapter complexes are responsible for the subcellular localisation and activation of signalling molecules such as PLCγ and small GTPases. Previous work in the laboratory (Melendez *et al.*, 1998a) has demonstrated that FcγRI and FcγRII couple to distinct signalling pathways in IFN-γ primed U937 cells. Thus, FcγRII couples to the activation of PLC-γ, while FcγRI couples to PC-PLD activation. To elucidate the molecular mechanisms behind these differences, adapter protein complexes likely to be involved in Fcγ receptor signal transduction have been analysed.

5.2.1.1. SLP-76 and PLCγ1 associate with FcγRII following receptor aggregation

In FcεRI and T-cell antigen receptor signalling, the adapter protein SLP-76 plays a central role in coupling receptor aggregation to PLCγ activation. As SLP-76 is also expressed in monocytes and macrophages, this adapter protein is likely to play a role in Fcγ receptor mediated PLCγ activation. Here, a role for SLP-76 in signal transduction by either FcγRI or FcγRII was investigated.

IFN-γ primed U937 cells were activated by aggregation of either FcγRI or FcγRII with monoclonal antibodies (section 2.3.1.2) for 2 minutes. Following the preparation of cell lysates, SLP-76 was immune precipitated and analysed by Western blot (Figure 5.1). Figure 5.1A demonstrates that aggregation of either FcγRI or FcγRII induces high levels of protein tyrosine phosphorylation in total cell lysates. Analysis of SLP-76 immune precipitates with anti-phosphotyrosine antibodies indicated that SLP-76 becomes phosphorylated following aggregation of either FcγRI or FcγRII (Figure 5.1B). However, the appearance of a broad phosphotyrosine band at around 40 kDa in SLP-76 precipitates following specific aggregation of FcγRII was intriguing. This band did not appear in SLP-76 immune precipitates following specific aggregation of FcγRI in the same cells. As

FcγRII itself runs as a broad band at around 40kDa, this suggested that SLP-76 may interact directly with the receptor. As Western blotting antibodies against FcγRIIa are not available, it was not possible to detect FcγRII directly. Instead, FcγRII immune complexes were immune precipitated via the crosslinking goat anti-mouse antibody, following FcγRII activation. These complexes contain phosphorylated FcγRIIa and allow direct comparison with the 40 kDa band present in SLP-76 immune precipitates. Figure 5.1B clearly demonstrates exact co-migration of phosphorylated FcγRIIa in immune complex precipitates with the phosphorylated band found in SLP-76 immune precipitates. Further to this, reprobing the FcγRIIa precipitates with SLP-76 antibodies, reveals the presence of SLP-76 in FcγRII immune complexes (Figure 5.1C). Western blots of the SLP-76 immune precipitates from control cells, and following aggregation of either FcγRI or FcγRII were reprobed for SLP-76, to ensure equal loading of immune precipitates (Figure 5.1C). To examine proteins associating with SLP-76 after receptor aggregation, SLP-76 immune precipitate blots were also reprobed for γ-chain, PLCγ1 and Grb2 (Figure 5.1C). The γ-chain was readily detectable in cell lysates but no γ-chain was detectable in SLP-76 precipitates (Figure 5.1C). This indicates that the γ-chain does not associate with SLP-76 following aggregation of either FcγRI or FcγRII in these cells.

SLP-76 can interact with the SH3 domains of effector proteins, such as PLCγ1, via its central proline rich region (Kane *et al.*, 2000). Reprobing SLP-76 immune precipitates with anti-PLCγ1 antibodies reveals that PLCγ1 can be detected in immune precipitates even in unstimulated cells, indicating a constitutive interaction between PLCγ1 and SLP-76. This constitutive interaction remains unaffected by FcγRII aggregation (Figure 5.1C). Interestingly, aggregation of FcγRI appears to decrease the association of SLP-76 with PLCγ1. This data is consistent with FcγRI failing to couple to PLCγ1 activation.

As the membrane associated adapter protein, LAT, has been shown in other systems to anchor SLP-76 and PLCγ1 at the plasma membrane to allow activation, blots were also probed with polyclonal anti-LAT antibodies. LAT was readily detectable in cell lysates but not in SLP-76 immune precipitates or FcγRII immune complexes (data not shown).

Taken together, these data imply that FcγRII recruits both SLP-76 and PLCγ1 to immune complexes at the plasma membrane. The fact that SLP-76 and PLCγ1 are associated even in resting cells suggests that SLP-76 and PLCγ1 may be recruited as a preformed complex. Conversely, SLP-76 does not appear to associate with the γ-chain signalling subunit of FcγRI. Further, FcγRI appears to decrease the association of SLP-76 with PLCγ1. These data provide a mechanism for the previous observation that FcγRII, but not FcγRI, couples to PLCγ1 activation.

5.2.1.2. FcγRI but not FcγRII induces the association of SLP-76 with Grb2

Further to PLCγ activation, SLP-76 plays a central role in the activation of small G-protein coupled pathways. Central to this activation is the ubiquitous Grb2 adapter protein. As a tyrosine phosphorylated bands of around the correct size for Grb2 appear in SLP-76 immune precipitates, blots were reprobed with mouse anti-Grb2 monoclonals. Figure 5.1C (bottom panel) reveals that FcγRI, but not FcγRII, aggregation induces the association of Grb2 with SLP-76 (Figure 5.1C). The slightly larger 25 kDa band appearing in SLP-76 precipitates from FcγRII stimulated cells, is not Grb2 but rather corresponds to anti-FcγRII mAb light chain (this band is detected directly by the secondary anti-mouse:HRP). The intense band present in the FcγRII immune precipitate also corresponds to the anti-FcγRII mAb light chain. The appearance of Grb2 in SLP-76 immune precipitates following FcγRI aggregation implies that SLP-76 mediates distinct responses for FcγRI and FcγRII.

5.2.1.3. FcγRI and FcγRII induce the formation of distinct Shc complexes

The adapter proteins, Grb2 and Shc, play a central role in the activation of the small GTPases, Ras and Rac, by localizing guanine nucleotide exchange factors at the plasma membrane. Here, a role for these adapter proteins in FcγRI and FcγRII signal transduction was investigated. IFN-γ primed U937 cells were stimulated via FcγRI or FcγRII for 2 minutes using mAbs, and cell lysates prepared as previously described (section 2.3.3). Attempts to precipitate Grb2 from cell lysates proved unsuccessful. However, Shc was successfully immune precipitated from cell lysates with a monoclonal

anti-Shc antibody. Immune precipitates were then subjected to Western blot analysis (section 2.3.6 and sections therein). Anti-phosphotyrosine analysis of Western blots reveals the presence of multiple tyrosine phosphorylated proteins in Shc immune precipitates following aggregation of either FcγRI or FcγRII (Figure 5.2A). No tyrosine phosphorylated bands were detected in Shc precipitates from unstimulated cell lysates. Detecting the phosphorylation of Shc itself is hindered by crossreactivity of the secondary, anti-mouse:HRP conjugate with the mouse anti-Shc precipitating antibody (Fig 5.2A - indicated by IgG).

The Shc immune precipitates were reprobed with various specific antibodies to signalling proteins. This allowed the identification of the major phosphotyrosine containing proteins in these Shc immune precipitates. Thus, in cells following specific aggregation of FcγRI, Shc forms complexes with p145 SHIP, p120 Cbl, SLP-76 and Grb2 (Figure 5.2B), and these proteins are tyrosine phosphorylated (Figure 5.2A). In cells following the specific aggregation of FcγRII, the Shc complex also contains SHIP, Cbl and SLP-76 as tyrosine phosphorylated proteins (Figure 5.2B). However, no Grb2 could be detected (Figure 5.2B). As well as lacking Grb2, the stoichiometry of Shc complexes induced by FcγRII differ from those induced by FcγRI. Thus, FcγRI induced Shc complexes contain significantly higher levels of Cbl and SLP-76 than those induced by FcγRII. Levels of SHIP in Shc precipitates however are almost identical. None of these proteins were detectable in Shc immune precipitates from resting cells, indicating that these interactions are induced by receptor aggregation. Reprobing blots with rabbit polyclonal anti-Shc antibodies indicates equal loading of Shc in the immune precipitates (Figure 5.2 B).

5.2.1.4. FcγRI, but not FcγRII induces the formation of Shc-Grb2-Sos complexes

Shc has been implicated in the recruitment of the GEFs, Sos and Vav, to activate Ras and Rac1 respectively. Shc immune precipitates were therefore probed for the presence of Sos (GEF for Ras) and Vav (GEF for Rac1). Figure 5.3 indicates the presence of both Sos and Vav in Shc immune precipitates from unstimulated cells. FcγRI aggregation induced an approximate four fold increase in Sos associated with Shc complexes. As FcγRI also

induces the association of Shc with Grb2, this data supports a role for Shc-Grb2-Sos complexes in FcγRI signal transduction. No significant increase in Sos levels associated with Shc was observed in response to FcγRII aggregation, consistent with the absence of Grb2 in these complexes (Figure 5.3). Interestingly, both FcγRI and FcγRII aggregation induced an approximate two fold increase in Vav in Shc immune precipitates. These data together imply that FcγRI and FcγRII might couple to different patterns of small GTPase activation.

5.2.2. Regulation of Ras and Rac by FcγRI and FcγRIIa

The finding that FcγRI and FcγRII induce the formation of distinct adapter protein complexes with the effectors, Sos and Vav, led to experiments to investigate the activation of the small GTPases, Ras and Rac1. The ability of FcγRs to activate Ras and Rac was assessed using Ras and Rac assay kits (section 2.3.7). Here, agarose conjugated fusion proteins comprising either the Ras binding domain of Raf-1 or the Rac1 binding domain of PAK (a Rac1 effector) are used to precipitate active Ras-GTP and active Rac1-GTP, respectively. As these fusion proteins do not bind the inactive GDP bound forms of Ras or Rac1, the quantity of GTPase precipitated, as assessed by Western blot, is a measure of activity.

5.2.2.1. Both FcγRI and FcγRII activate Ras and Rac1

The ability of FcγRI and FcγRII to activate Ras and Rac1 was assessed in IFN-γ primed U937 cells by aggregating specific receptors with monoclonal antibodies (section 2.3.1.2). Following preparation of cell lysates, active Ras and Rac1 were precipitated as described above (Figure 5.4). Control lanes indicate that simply warming the cells to 37°C induces some activation of Ras and Rac1. Aggregation of FcγRI led to a substantial increase in Ras-GTP above control within 1 minute which was increased further by 3 minutes (Figure 5.4A). FcγRI also induced a transient small increase in Rac1 activity with maximal activity observed at 1 minute. FcγRII aggregation also induced the activation of both Ras and Rac1 although the increase in Ras was much lower than that observed for FcγRI (Figure 5.4B). The apparently enhanced Ras activity in the 3 minute control lane

(Figure 5.4B - control 3) is a result of longer exposure times required to detect Ras activity induced by FcγRII. Rac1 activation induced by FcγRII follows a similar pattern to that observed for FcγRI.

5.2.2.2. Activation of Ras and Rac by FcγRI and FcγRII is downstream of PI 3-kinase activation

As activation of small GTPases often lies downstream of PI 3-kinase, the effect of PI 3-kinase inhibitors on Ras and Rac1 activation was investigated. Preincubation of cells with the PI 3-kinase inhibitors LY294002 (250 μM) and wortmannin (50 nM) inhibited the majority of Ras and Rac1 activation induced by FcγRI aggregation (Figure 5.5A). FcγRII induced Ras activity was also inhibited by preincubation of cells with LY294002 (250 μM) and wortmannin (50 nM) (Figure 5.5B). FcγRII induced Rac1 activation was less sensitive to PI 3-kinase inhibitors although LY294002 (250μM) substantially reduced activation. Preincubation of cells with wortmannin was found to only slightly decrease FcγRII mediated Rac1 activation.

5.2.2.3. Ras and Rac activation does not require activation of p85-dependent PI 3-kinase

Previous work in the laboratory has demonstrated that FcγRI couples to both tyrosine kinase dependent and G-protein coupled PI 3-kinase isoforms, to generate a prolonged PIP_3 response (Melendez *et al.*, 1998c). Conversely, FcγRII couples solely to tyrosine kinase dependent PI 3-kinase to give a short PIP_3 response (Melendez *et al.*, 1998c) (unpublished observations). Overexpression of a dominant negative isoform of the p85 subunit (Δp85) of the tyrosine kinase dependent PI 3-kinase blocks activation by preventing recruitment of the catalytic p110 subunit to phosphorylated ITAMs (Hara *et al.*, 1994). Thus, overexpression of Δp85 in U937s abolished PIP_3 production in response to FcγRII aggregation. As Δp85 has no effect on G-protein coupled PI 3-kinase, overexpression of Δp85 only delays the production of PIP_3 (Melendez *et al.*, 1998c).

Expression of dominant negative Δp85 in the U937:Δp85 cell line was induced with IPTG (described in section 2.2.1.2). Overexpression of the truncated Δp85 was confirmed by Western blot (Figure 5.6), which shows expression of the truncated Δp85 only after induction with IPTG. Comparison of band intensity indicates that dominant negative Δp85 is expressed at approximately 20 fold higher levels than endogenous p85. As insulin is reported to activate only p85 dependent PI 3-kinase in U937 cells, insulin was used as a control (Hara *et al.*, 1994). Either FcγRI or FcγRII was aggregated for 1 minute in either control U937 cells or U937 cells induced to overexpress Δp85. Alternatively, cells were incubated for 1 minute with insulin (10 μg/ml). Expression of Δp85, rather than blocking FcγRI or FcγRII mediated activation of Ras, resulted in a small enhancement of induced activity in all cases (Figure 5.7). Likewise, insulin induced Ras activation was slightly enhanced by overexpression of Δp85. Expression of Δp85 had little effect on Rac1 activation by either FcγRI or FcγRII. Insulin only induced very low levels of Rac1 activation at the time point examined making it difficult to assess the effects of Δp85 expression here. In order to assess whether overexpression of Δp85 affected the ability of Fcγ receptors and insulin to induce tyrosine phosphorylation, levels of phosphotyrosine in cell lysates were assessed by Western blot (section 2.3.6.1). Comparison of phosphotyrosine responses in cell lysates indicates that Δp85 also slightly enhances Fcγ receptor and insulin induced tyrosine phosphorylation (Figure 5.7 - bottom panel). In addition, close examination of the phosphotyrosine blots indicates that a band of the correct size for Δp85, present only in the IPTG induced samples, is tyrosine phosphorylated in response to both FcγRI and FcγRII aggregation (Figure 5.7 - indicated Δp85). Taken together, these data imply that Ras and Rac activation do not depend on the activation of the tyrosine kinase dependent PI 3-kinase. Furthermore, as both Ras and Rac activation are inhibited by LY294002 and wortmannin, but not by Δp85 expression, these data also imply that other PI 3-kinase isoforms lie upstream of Ras and Rac.

5.2.3. Regulation of MAP Kinase activation by FcγRI and FcγRIIa

Next, the coupling of FcγRI and FcγRII to the MAPK signalling cascades was examined. To investigate whether FcγRI and FcγRII couple to different MAPK pathways in IFN-γ

primed U937 cells, specific receptors were aggregated and MAPK phosphorylation was measured by Western analysis (section 2.3.6.2). Cells were harvested at given time points after receptor aggregation and lysed. Western blots of cell lysates were probed with antibodies which specifically recognise dual phosphorylated active MAPKs as described in materials and methods (section 2.3.6.2). As Ras and Rac activation were found to be influenced by the inhibitors wortmannin and LY294002, their effect on activation was also examined.

5.2.3.1. FcγRI and FcγRII activate ERK1 and ERK2 downstream of PI 3-kinase

Aggregation of FcγRI induced the rapid transient phosphorylation of both p42 and p44 ERK within 3 minutes of receptor aggregation (Figure 5.8A - top panel). Levels of phosphoERK decreased partially 30 minutes after receptor aggregation. ERK activation by FcγRI was almost entirely abolished by preincubation with the PI 3-kinase inhibitor LY294002 (250 μM). Wortmannin (50 nM), a structurally distinct PI 3-kinase inhibitor, also inhibited ERK activation but to a lesser extent than LY294002. Blots were striped and reprobed with antibodies recognizing all forms of ERK to ensure equivalent loading between lanes (Figure 5.8A - bottom panel).

Aggregation of FcγRII with mAbs (clone IV3) also induced the phosphorylation of both p42 and p44 ERK though the response appeared slower than that observed for FcγRI (Figure 5.8B - top panel). Peak phosphorylation was observed 10 minutes following aggregation. By 30 minutes, phosphorylation had returned to near basal levels. ERK activition was completely abolished by preincubation with LY294002 (250μM). Wortmannin (50nM) was also found to inhibit ERK phosphorylation, though to a lesser extent than LY294002. Blots were stripped and reprobed with antibodies recognizing all forms of ERK to ensure comparable levels were present in each sample (Figure 5.8B bottom panel).

5.2.3.2. FcγRI and FcγRII activate p38 MAPK

Dual phosphorylation of p38 in response to FcγRI and FcγRII aggregation was assessed as for ERK (section 2.3.6.2). Levels of phospho-p38 were substantially elevated at 3 minutes and 10 minutes after FcγRI aggregation (Figure 5.9A). After 30 minutes levels of phospho-p38 had returned to basal levels. The PI 3-kinase inhibitor, LY294002, reduced FcγRI induced p38 phosphorylation at earlier time points (3 minutes and 10 minutes). However, 30 minutes following FcγRI aggregation, inhibition of PI 3-kinase with LY294002 appeared to enhance the levels of phospho-p38 above untreated cells. Inhibition of PI 3-kinase with wortmannin had little effect on phosphorylation of p38 and a reduction was only observed at 10 minutes post receptor aggregation.

FcγRII aggregation also induced phosphorylation of p38 though to a lesser extent than FcγRI (Figure 5.9B). Also, phosphorylation of p38 in response to FcγRII showed slower kinetics than FcγRI, with levels of phospho-p38 remaining above basal levels 30 minutes after FcγRII aggregation. Surprisingly, treatment of cells with LY294002, rather than inhibiting p38 activation, caused a substantial increase in phosphorylation of p38, particularly 30 minutes after receptor aggregation. Wortmannin, as with FcγRI had little effect on p38 activation except at the 10 minute timepoint where inhibition of p38 phosphorylation was almost complete.

5.2.3.3. ERK and p38 activation do not require p85-dependent PI 3-kinase activation

As described earlier, FcγRI and FcγRII couple to distinct patterns of PI 3-kinase activation. Thus, FcγRI couples to both tyrosine kinase dependent PI 3-kinase and G-protein coupled PI 3-kinase, whereas FcγRII couples solely to tyrosine kinase dependent PI 3-kinase isoform. To investigate whether differences in the sensitivity of ERK and p38 activation by FcγRI and FcγRII to PI 3-kinase inhibitors was a result of these differences in PI 3-kinase isoform activation, the effect of Δp85 overexpression on MAPK activation was assessed. Figure 5.10 shows the activation profiles of ERK and p38 in response to FcγRI aggregation in uninduced U937:Δp85 and induced U937:Δp85. These data demonstrate that overexpression of Δp85 does not block either ERK or p38

activation by either FcγRI (Figure 5.10) or FcγRII. In fact ERK and p38 phosphorylation appeared to be enhanced by overexpression of Δp85 in all cases. Reprobing blots for total ERK and total p38 demonstrate comparable loading of cell lysates (Figure 5.10).

5.2.3.4. FcγRI activates JNK

As for ERK and p38, coupling of FcγRI and FcγRII to the JNK pathway was assessed with phosphorylation state specific antibodies (section 2.3.6.2). Figure 5.11A demonstrates that FcγRI aggregation induces JNK phosphorylation, peaking at 10 minutes after aggregation and returning to near basal levels by 30 minutes. Conversely, FcγRII failed to induce detectable JNK phosphorylation over the timecourse examined (Figure 5.11A). Figure 5.11B demonstrates that FcγRI stimulated JNK phosphorylation is inhibited to an equal degree by both LY294002 and wortmannin. Figure 5.11C indicates that overexpression of Δp85, also does not block FcγRI induced JNK activation. Rather, expression of Δp85 enhances levels of phospho-JNK induced by FcγRI, in line with data presented earlier.

In summary, both FcγRI and FcγRII are coupled to the ERK and p38 MAPK pathways. However, while both receptors coupled to comparative levels of ERK activation, only FcγRI induced an increase in Shc-Grb2-Sos complexes and substantial Ras activation. These data might suggest differences in the ERK activation pathway utilised by FcγRI and FcγRII. Interestingly, only FcγRI appears to couple to the activation of JNK.

5.3. Discussion

The adapter proteins, SLP-76 and LAT, are essential for the activation of PLCγ in T-cell antigen receptor signalling and FcεRI signalling in mast cells (Revied in Kane *et al.*, 2000, Turner & Kinet, 1999). Here, LAT recruits PLCγ1 to the plasma membrane while SLP-76 is essential for activation of PLCγ1 by Tec family kinases. The mechanism linking Fcγ receptors to PLCγ activation, however, remains unclear. In IFN-γ primed U937 cells, we have established that FcγRII is coupled to the activation of PLCγ1. Conversely, aggregation of FcγRI fails to activate this pathway. Data presented here provides a potential explanation for these findings. In IFN-γ primed U937 cells, SLP-76 and PLCγ1 were found to be constitutively associated. Following aggregation of FcγRII, SLP-76 is tyrosine phosphorylated and associates with a number of phosphotyrosine containing proteins. The most prominent of these tyrosine phosphorylated proteins is FcγRII itself. Further, both SLP-76 and PLCγ1 can be detected in FcγRII signalling complexes. LAT, which anchors PLCγ to the plasma membrane in TCR and FcεRI signalling, could not be detected in association with either FcγRII or SLP-76. These data provide evidence that FcγRII might directly recruit SLP-76-PLCγ1 complexes to the plasma membrane, where activation can take place. Interestingly, aggregation of FcγRI under identical conditions also results in the tyrosine phosphorylation of SLP-76 and its association with multiple tyrosine phosphorylated proteins. However, neither the γ-chain (the signalling subunit for FcγRI and also FcεRI) nor FcγRII could be found in SLP-76 complexes following FcγRI aggregation. These data are consistent with the previous finding that FcγRI does not couple to the activation of PLCγ in IFN-γ primed U937 cells. It is tempting to speculate that the ability of FcγRIIa, but not γ-chain, to recruit SLP-76-PLCγ1 complexes is a result of the atypical ITAM motif of FcγRIIa (section 1.2.1.1).

Interestingly, the constitutive association between SLP-76 and PLCγ, observed in resting U937 cells appeared to be decreased following aggregation of FcγRI. Further, FcγRI aggregation was found to induce the association of SLP-76 with Grb2. As SLP-76 is reported to interact with the SH3 domain of Grb2 via its proline rich domain, this raises

the possibility that the SH3 domains of Grb2 and PLCγ1 might compete for binding to the proline rich domain of SLP-76. This hypothesis is under active investigation.

Though FcγRI fails to couple to PLCγ, FcγRI aggregation was found to induce the tyrosine phosphorylation of SLP-76 and its association with the adapter proteins, Shc and Grb2. SLP-76, Shc and Grb2 have been implicated in the activation of the small GTPases, Ras and Rac1 (section 1.2.5.2). A potential role for these adapters was examined in the coupling of FcγRI to the activation of Ras and Rac1. Consistent with a role for these adapters in FcγRI mediated Ras activation, levels of Sos associated with Shc complexes were substantially increased in response to FcγRI aggregation. Further, previous data (Cameron & Allen, 1999) indicates that FcγRI induces the translocation of Shc to the plasma membrane in response to receptor aggregation. In contrast to FcγRI, aggregation of FcγRII, did not induce the association of Grb2 with either Shc or SLP-76. In addition, no increase in Sos levels associated with Shc was detected. Consistent with these findings, FcγRI was found to couple more efficiently to Ras than FcγRII. Both FcγRI and FcγRII induced comparable increases in Vav association with adapter complexes. Patterns of Rac activation for FcγRI and FcγRII were also similar.

A major response to Ras activation is the activation of Raf-1 and the subsequent coupling to MEK and ERK (section 1.2.6.2). Despite apparently significant differences in levels of Ras activation induced by FcγRI and FcγRII, both receptors were found to couple to comparable levels of ERK activation, as assessed by analysis of phosphoERK levels. Recent reports have implicated Ras independent pathways in the activation of ERK by Fcγ receptors. Firstly, in human neutrophils, aggregation of either FcγRIIa or FcγRIIIb (the latter not expressed by U937 cells) induces ERK activation, independent of the formation of Shc-Grb2-Sos complexes (Hazan-Halevy *et al.*, 2000). Secondly, Sanchez-Mejorda et al show that Fcγ receptor mediated activation of ERK in THP-1 and U937 cells is largely unaffected by expression of dominant negative Ras or Raf-1 (Sanchez-Mejorada & Rosales, 1998). The U937 cells used in this study were not primed with IFN-γ and will thus express relatively low levels of FcγRI in comparison to the cells used here. Further, FcγRI is reported not to associate with the γ-chain in undifferentiated U937 cells (van de Winkel & Capel, 1993). These reports are consistent with the findings

here that FcγRIIa is able to activate ERK in the absence of a robust Ras activation. Taken together with the finding that FcγRI induces the formation of Shc-Grb2-Sos complexes and robust Ras activation, these data imply that FcγRI and FcγRII may couple to the activation of ERK via distinct signalling pathways.

FcγRI and FcγRII induced comparable increases in Vav associated with Shc adapter complexes. Patterns of Rac activation for FcγRI and FcγRII were also similar. As Rac1 has been found to lie upstream of p38 and JNK in other systems, the coupling of FcγRI and FcγRII to p38 and JNK was assessed. FcγRI was found to couple to both p38 and JNK phosphorylation, while FcγRII was found to induce a comparably modest induction of p38 phosphorylation and little or no JNK response. The reasons for these differences are not clear.

Interestingly, Fcγ receptor aggregation on murine macrophages has been found to activate ERK, p38 and JNK (Rose *et al.*, 1997). Further, FcεRI aggregation in mouse bone marrow derived mast cells also couples to all three MAP kinase cascades (Ishizuka *et al.*, 1997, Ishizuka *et al.*, 1999). As murine Fcγ receptors and FcεRI couple to the activation of signal transduction pathways via the γ-chain, these data indicate that the γ-chain ITAM is capable of activating all three MAPK cascades. Consistent with a conserved function for the γ-chain in the regulation of human MAPK activation, FcγRI in IFN-γ primed U937 cells also activates ERK, p38 and JNK.

Also of interest, the activation patterns of the MAP kinases by FcγRIIa closely follows those reported for the phagocytosis of IgG opsonised bacteria by neutrophils (McLeish *et al.*, 1998). Here, Fcγ receptors couple to the activation of ERK and p38, but not JNK activation. Neutrophils express FcγRIIa and FcγRIIIb, though as FcγRIIb is GPI anchored, and cannot associate with γ-chain, signal transduction is thought to be governed primarily by FcγRIIa (Hazan-Halevy *et al.*, 2000). It seems likely, therefore, that the same pathway is used by FcγRIIa in both neutrophils and monocytes. Thus, FcγRIIa might provide a γ-chain independent pathway for immune complexes to mediate a distinct MAP kinase response. This is particularly intriguing as FcγRIIa is a human specific receptor.

The significance of differences in the MAP kinase profiles activated by FcγRI and FcγRII is not clear. P38 has been implicated in mediating activation of the respiratory burst (Herlaar & Brown, 1999, Hazan-Halevy *et al.*, 2000) and thus this pathway may be expected to lie downstream of both FcγRI and FcγRII. The differences in JNK activation will likely reflect differences in transcriptional regulation by FcγRI and FcγRII. ERK, p38 and JNK have been variously implicated in the regulation of inflammatory cytokine production though responses appear to vary greatly between different systems and cell types (Paul *et al.*, 1997, Ip & Davis, 1998, Herlaar & Brown, 1999). The transcriptional response mediated by FcγRI and FcγRII remain to be determined.

The effects of the PI 3-kinase inhibitors wortmannin and LY294002 on activation of the MAP kinase cascades raises some intriguing points. ERK activation induced by either FcγRI or FcγRII was inhibited by LY294002 and to a lesser extent by wortmannin. The effects of PI 3-kinase inhibitors on p38 phosphorylation are clearly complex. The data presented imply that PI 3-kinase may play important positive and negative regulatory roles in the regulation of p38 activity. Further work is required here to establish the role of PI 3-kinase in the regulation of this pathway. As MAP kinase pathways are also regulated by PKC, the effect of PKC inhibitors on Fcγ receptor mediated MAP kinase activation is also under investigation (Ueda *et al.*, 1996, Cote-Velez *et al.*, 1999, McLeish *et al.*, 1998).

As inhibitors may have additional effects, the role of PI 3-kinase was examined using a dominant negative isoform of the p85 subunit of p85 dependent PI 3-kinase. Overexpression of this dominant negative subunit abolishes PIP_3 production in response to insulin (Hara *et al.*, 1994) or FcγRII aggregation and delays PIP_3 production in response to FcγRI crosslinking (Melendez *et al.*, 1998c). Surprisingly however, overexpression of Δp85 did not inhibit, but rather slightly enhanced, Ras, Rac, MAPK and phospho-tyrosine responses induced by both FcγRI and FcγRII. In particular, the fact that FcγRII induced ERK, Ras and Rac responses were inhibited by LY294002 and wortmannin, but not Δp85 expression, implies a dependence on an alternative PI 3-kinase isoform (section 1.2.3.1).

We have previously demonstrated that in IFN-γ primed U937 cells FcγRII, but not FcγRI couples to the activation of PLCγ1 (Melendez *et al.*, 1998a). I show here that FcγRI and FcγRII couple to the assembly of different adapter protein scaffolds providing a potential mechanism for the differences in PLCγ activation observed. Following on from differences in adapter protein complexes FcγRI and FcγRII were shown to be coupled to distinct activation patterns of the MAPK cascades. Taken together, these data imply that FcγRI and FcγRII will induce distinct patterns of transcription. Future work will attempt to identify specific transcription factors and genes targeted by the two receptors with particular focus on the production of inflammatory cytokines.

Figure 5.1 SLP-76 associates with FcγRII following FcγRII aggregation. Cells were loaded with either anti-FcγRI mAb 22 or anti-FcγRII mAb KB61 at 4°C and receptors aggregated by the addition of goat anti-mouse IgG. Control cells were stimulated with goat anti-mouse IgG alone. Cells were warmed to 37°C for 2 minutes and cell lysates prepared. (A) cell lysates (10 μg/sample) were subjected to Western blot analysis with the antiphosphotyrosine mAb (4G10) (bottom panel). (B and C) SLP-76 was immune precipitated from cells lysates and precipitates were subjected to Western blot analysis. FcγRII immune complexes were also precipitated following FcγRII aggregation (indicated FcγRII IP on Western blots) via the goat anti-mouse crosslinker by the addition of anti-goat IgG conjugated agarose. 10 μg of cells lysate (lysate) was also included on Western blots. (B) Western blots were probed for phosphotyrosine with mAb 4G10. (C) Blots were stripped and reprobed for PLCγ, SLP-76, γ-chain and Grb2.

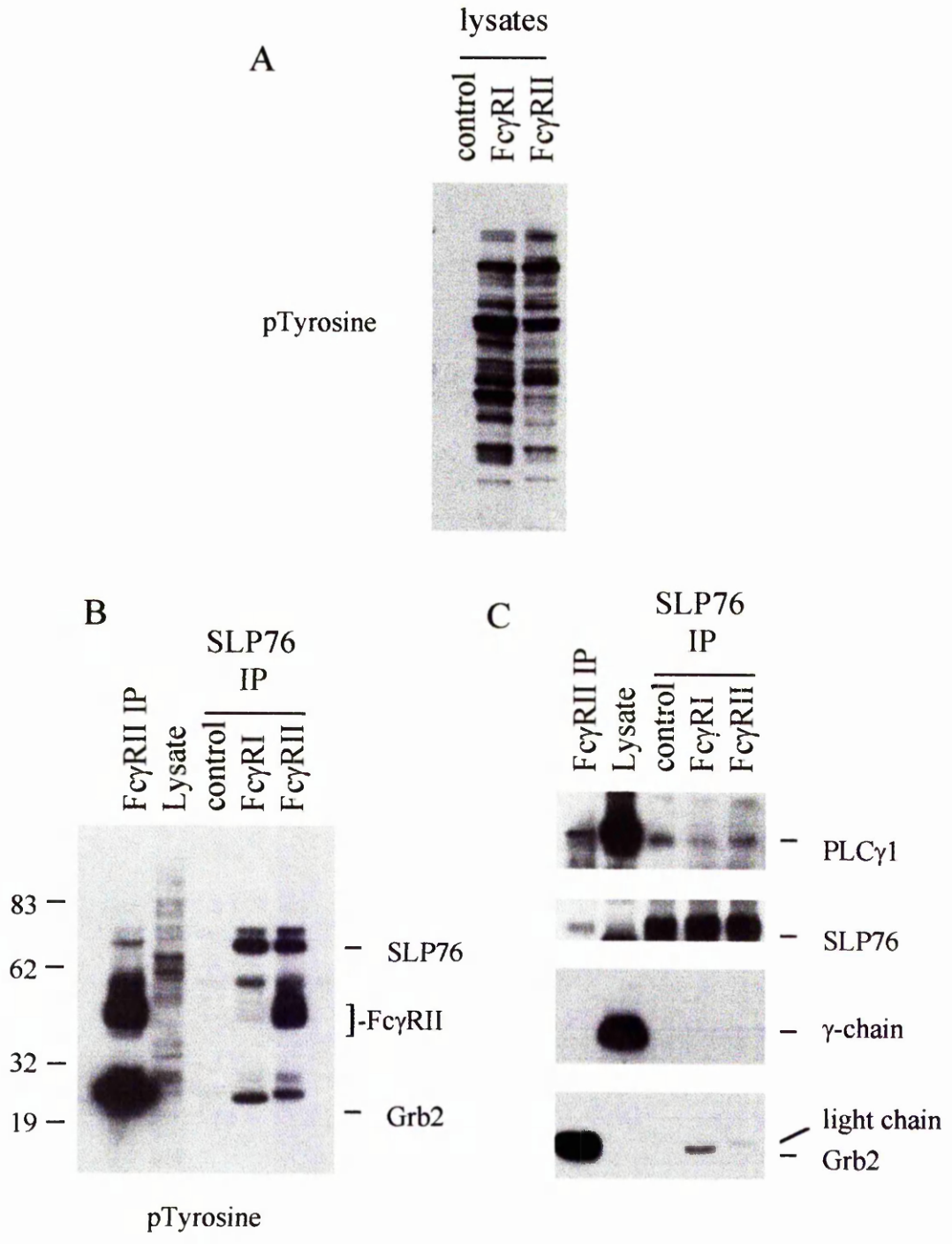

A
lysates
control
FcγRI
FcγRII
pTyrosine
B
SLP76 IP
FcγRII IP
Lysate
control
FcγRI
FcγRII
83
62
32
19
SLP76
]-FcγRII
Grb2
pTyrosine
C
SLP76 IP
FcγRII IP
Lysate
control
FcγRI
FcγRII
PLCγ1
SLP76
γ-chain
light chain
Grb2

Figure 5.2 FcγRI and FcγRII induce the formation of distinct Shc complexes.

Cells were loaded with either anti-FcγRI mAb 22 or anti-FcγRII mAb KB61 at 4°C and receptors aggregated by the addition of goat anti-mouse IgG. Control cells were stimulated with goat anti-mouse IgG alone. Cells were warmed to 37°C for 2 minutes and cell lysates prepared. Shc was immune precipitated from cell lysates and probed for the presence of tyrosine phosphorylated proteins with mAb 4G10 (A). Blots were stripped and reprobed (B) for SHIP, Cbl, SLP-76, Shc and Grb2 as indicated.

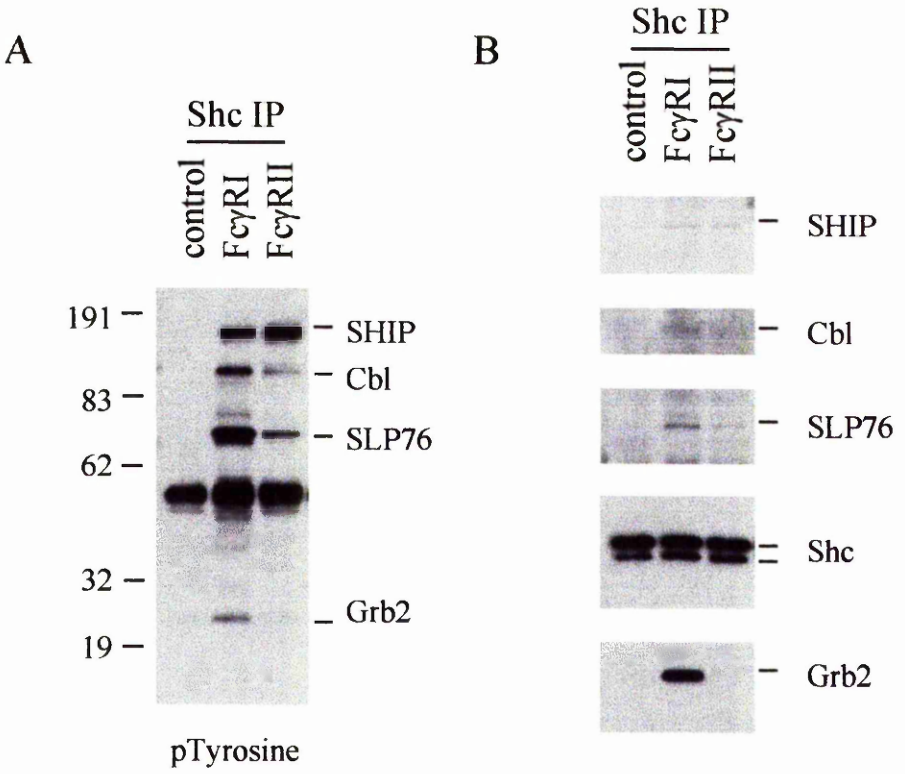

A
Shc IP
control
FcγRI
FcγRII
191
83
62
32
19
SHIP
Cbl
SLP76
Grb2
pTyrosine
B
Shc IP
control
FcγRI
FcγRII
SHIP
Cbl
SLP76
Shc
Grb2

Figure 5.3 Analysis of Shc-Sos and Shc-Vav complexes induced by aggregation of FcγRI and FcγRII.

Cells were loaded with either anti-FcγRI mAb 22 or anti-FcγRII mAb KB61 at 4°C and receptors aggregated by the addition of goat anti-mouse IgG. Control cells were stimulated with goat anti-mouse IgG alone. Cells were warmed to 37°C for 2 minutes and cell lysates prepared. Shc was immune precipitated from cells lysates and probed for the presence of Sos (top panel), Vav (middle panel) and Shc (bottom panel). The numbers beneath the Sos and Vav blots indicate relative intensity of bands (normalised to control) as calculated by densitometry.

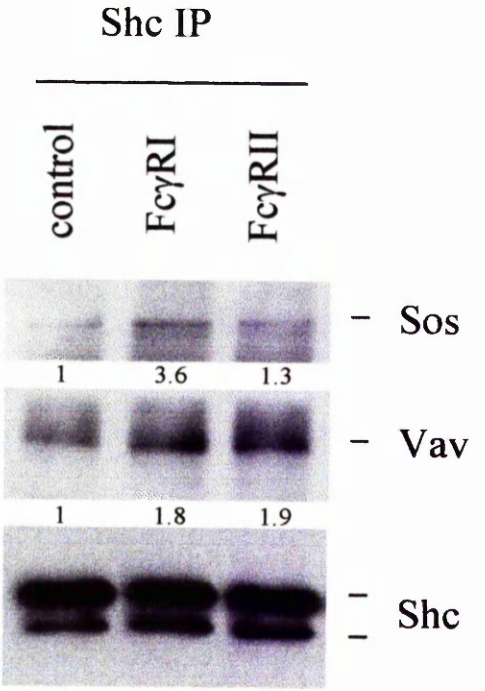
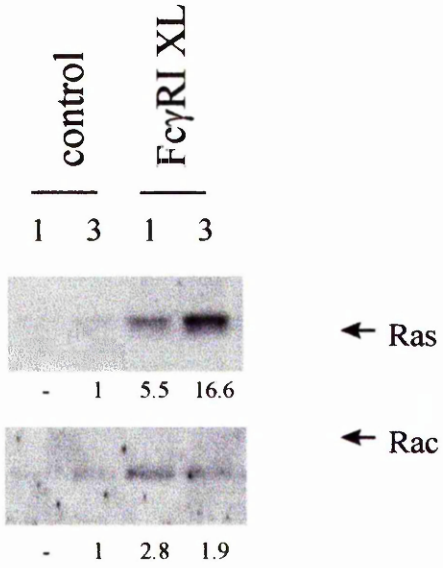

Shc IP
control
FcγRI
FcγRII
Sos
1
3.6
1.3
Vav
1
1.8
1.9
Shc

Figure 5.4 FcγRI and FcγRII activate Ras and Rac.

Cells were primed with IFN-γ for 24 hours prior to assays. Cells were loaded with either anti-FcγRI mAb 22 (A) or anti-FcγRII mAb KB61 (B) at 4°C and the receptors were aggregated by the addition of goat anti-mouse IgG. Control cells were stimulated with goat anti-mouse IgG alone. Cells were warmed to 37°C for the times indicated and Ras and Rac activity were assessed. Numbers beneath blots indicate the relative intensity of bands as assessed by densitometry, with the 3 minute control point of each experiment arbitrarily set as 1. No values are given for the 1 minute control points as bands were too faint for accurate quantitation.

A


control
FcγRI XL
1 3 1 3
Ras
- 1 5.5 16.6
Rac
- 1 2.8 1.9

B

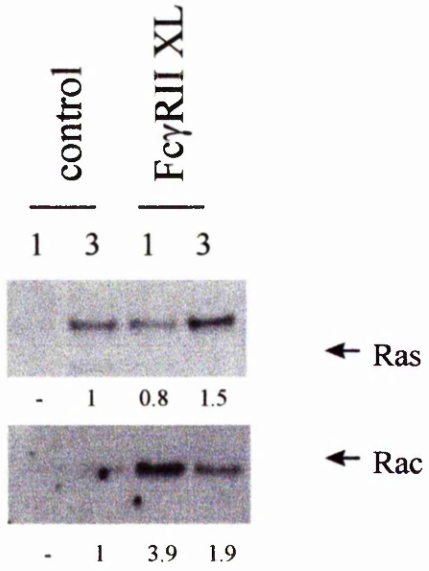
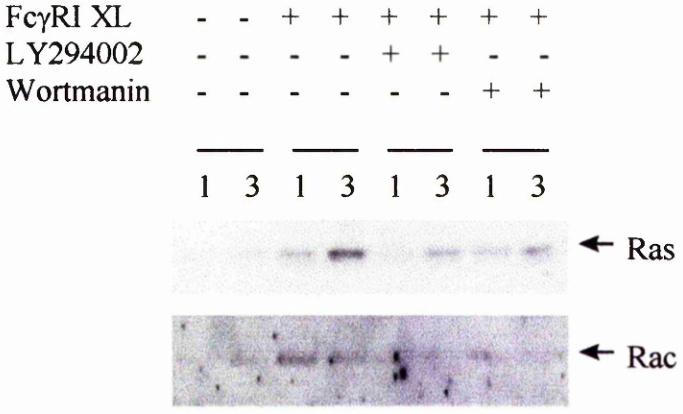

control
FcγRII XL
1 3 1 3
Ras
- 1 0.8 1.5
Rac
- 1 3.9 1.9

Figure 5.5 FcγRI and FcγRII activate Ras and Rac.

Cells were primed with IFN-γ for 24 hours prior to assays. Cells were loaded with either anti-FcγRI mAb 22 (A) or anti-FcγRII mAb KB61 (B) at 4°C and the receptors were aggregated by the addition of goat anti-mouse IgG. Control cells were stimulated with goat anti-mouse IgG alone. Cells were warmed to 37°C for the times indicated. To inhibit PI 3-kinase cells were incubated for 20 minutes prior to receptor aggregation with either 250 μM LY294002 or 50 nM wortmannin as indicated. Ras and Rac activity were assessed as described in section 2.3.7.

A


FcγRI XL - - + + + + + +
LY294002 - - - - + + - -
Wortmanin - - - - - - + +
1 3 1 3 1 3 1 3
Ras
Rac

B

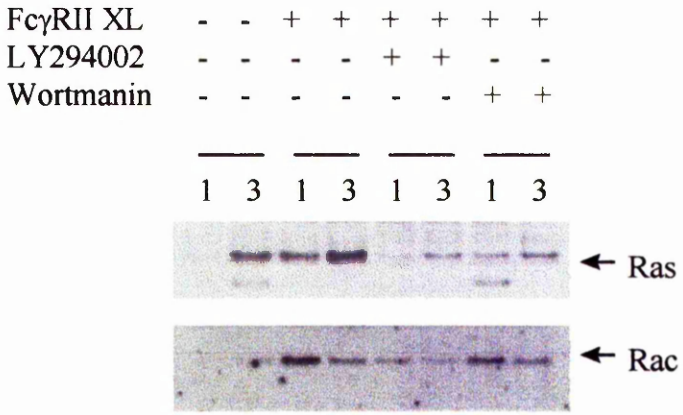

FcγRII XL - - + + + + + +
LY294002 - - - - + + - -
Wortmanin - - - - - - + +
1 3 1 3 1 3 1 3
Ras
Rac

Figure 5.6 Δp85 expression is induced in U937:Δp85 cells by IPTG

U937:Δp85 cells were cultured as described. Cells were primed with IFN-γ 24 hours prior to experiments. Δp85 expression was induced by 15 mM IPTG for 10 hours as described (section 2.2.1.2). Cells, with no addition of IPTG were used as a control. Cell lysates were prepared and 10 μg of each sample subjected to SDS-PAGE. Expression of Δp85 was assessed by Western blot analysis with anti-p85 PI 3-kinase rabbit polyclonal antibodies.

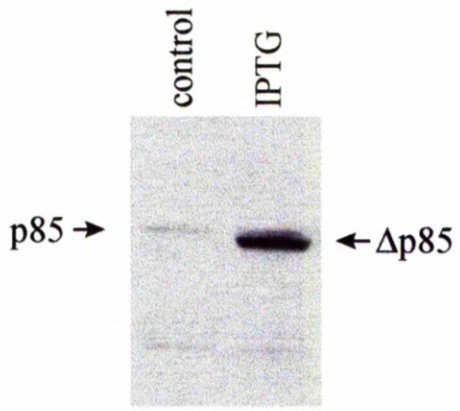

control
IPTG
p85
Δp85

Figure 5.7 FcγRI and FcγRII induced Ras and Rac activation are not dependent on p85 dependent PI 3-kinase.

U937:Δp85 cells were primed with IFN-γ 24 hours prior to experiments. To induce Δp85 expression, 15mM IPTG was added 10 hours prior to experiments. Cells were loaded with either anti-FcγRI mAb 22 or anti-FcγRII mAb KB61 at 4°C and receptors aggregated by the addition of goat anti-mouse IgG. Control cells were stimulated with goat anti-mouse IgG alone. Cells were warmed to 37°C for 1 minutes. Alternatively, cells were stimulated with 10 μg/ml insulin for 1 minutes as indicated. Ras (top panel) and Rac (middle panel) activity were assessed as described in section 2.3.7. To assess the effects of Δp85 expression on the induction of tyrosine phosphorylation, cell lysates (10 μg/sample) from the above described stimulations were subjected to Western blot analysis with mAb 4G10 (bottom panel).

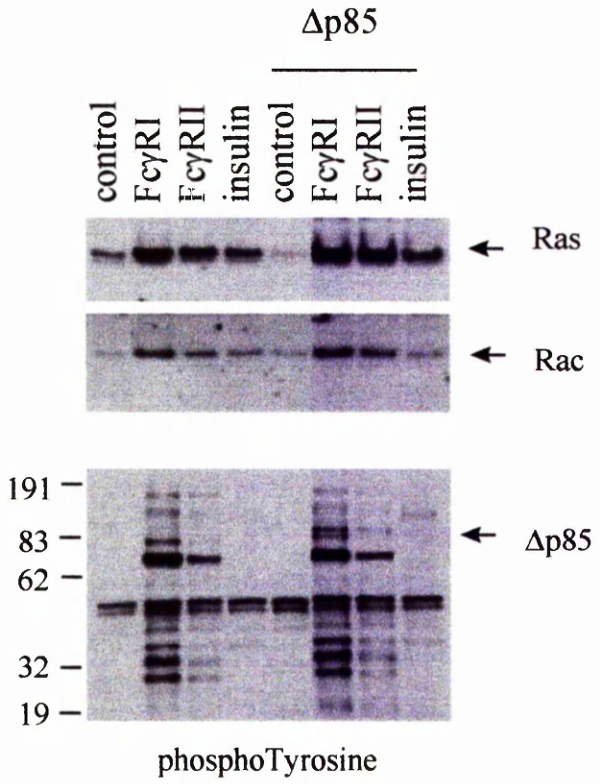
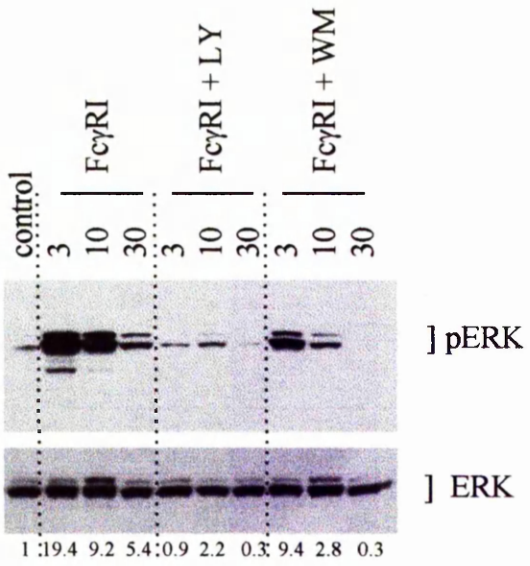

Δp85
control
FcγRI
FcγRII
insulin
control
FcγRI
FcγRII
insulin
Ras
Rac
191
83
62
32
19
Δp85
phosphoTyrosine

Figure 5.8 FcγRI and FcγRII activate ERK downstream of PI 3-kinase.
U937 cells were primed with IFN-γ for 24 hours prior to experiments. Cells were loaded with either anti-FcγRI mAb 22 (A) or anti-FcγRII mAb KB61 (B) at 4°C and receptors aggregated by the addition of goat anti-mouse IgG. Control cells were stimulated with goat anti-mouse IgG alone. Cells were warmed to 37°C for the times indicated. To inhibit PI 3-kinase, cells were pre-incubated with either LY294002 (indicated LY) or wortmannin (indicated by WM) for 20 minutes prior to receptor aggregation. Cells were warmed to 37°C for the times indicated (minutes) and cell lysates prepared. ERK phosphorylation was assessed in cell lysates (10 μg/sample) by Western blot analysis with polyclonal anti-phosphoERK antibodies (indicated pERK). Blots were stripped and reprobed with pan-ERK antibodies to control for equal loading (indicated ERK). Numbers beneath blots indicate the relative intensity of bands as assessed by densitometry, with the control point of each experiment arbitrarily set as 1. These data are corrected for equal loading.

A


control
3
10
30
FcγRI
3
10
30
FcγRI + LY
3
10
30
FcγRI + WM
] pERK
] ERK
1 19.4 9.2 5.4 0.9 2.2 0.3 9.4 2.8 0.3

B

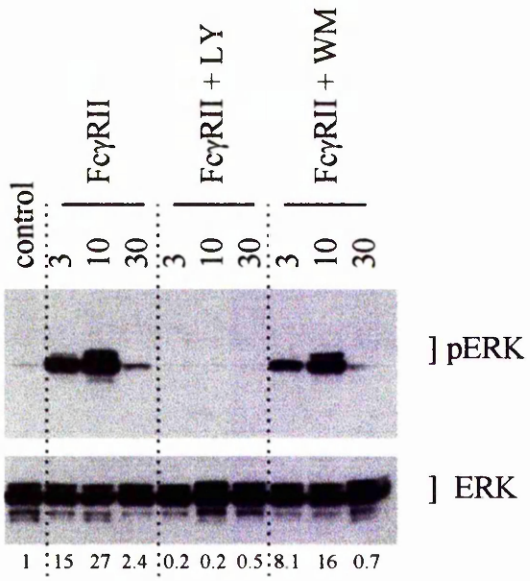

control
3
10
30
FcγRII
3
10
30
FcγRII + LY
3
10
30
FcγRII + WM
] pERK
] ERK
1 15 27 2.4 0.2 0.2 0.5 8.1 16 0.7

Figure 5.9 FcγRI and FcγRII activate p38 with different sensitivities to PI 3-kinase inhibition.

U937 cells were primed with IFN-γ for 24 hours prior to experiments. Cells were loaded with either anti-FcγRI mAb 22 (A) or anti-FcγRII mAb KB61 (B) at 4°C and receptors aggregated by the addition of goat anti-mouse IgG. Control cells were stimulated with goat anti-mouse IgG alone. Cells were warmed to 37°C for the times indicated. To inhibit PI 3-kinase, cells were pre-incubated with either LY294002 (indicated LY) or wortmannin (indicated by WM) for 20 minutes prior to receptor aggregation. Cells were warmed to 37°C for the times indicated (minutes) and cell lysates prepared. P38 phosphorylation was assessed in cell lysates (10 μg/sample) by Western blot analysis with polyclonal anti-phospho-p38 antibodies (indicated pp38). Blots were stripped and reprobed with pan-p38 antibodies to control for equal loading (indicated p38). Numbers beneath blots indicate the relative intensity of bands as assessed by densitometry, with the control point of each experiment arbitrarily set as 1. These data are corrected for equal loading.

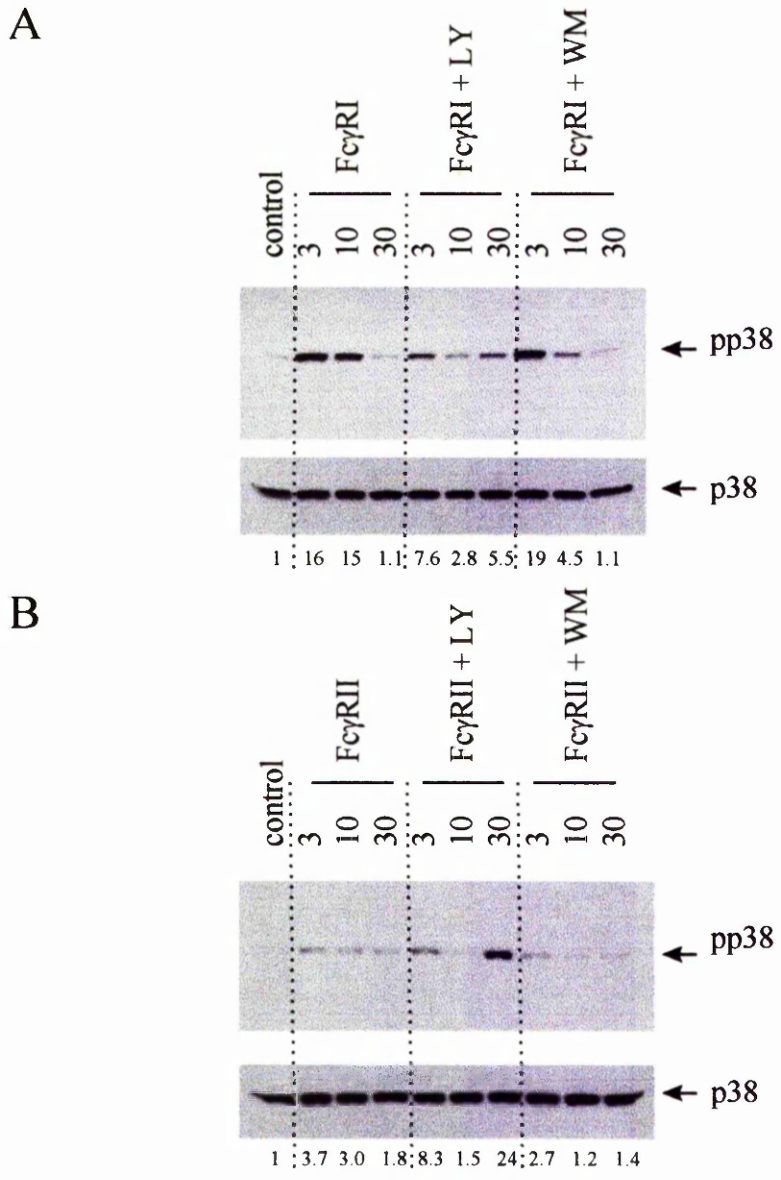

A
control
FcγRI
FcγRI + LY
FcγRI + WM
3
10
30
3
10
30
3
10
30
pp38
p38
1 16 15 1.1 7.6 2.8 5.5 19 4.5 1.1
B
control
FcγRII
FcγRII + LY
FcγRII + WM
3
10
30
3
10
30
3
10
30
pp38
p38
1 3.7 3.0 1.8 8.3 1.5 24 2.7 1.2 1.4

Figure 5.10 FcγRI induced ERK and p38 activation are not dependent on p85 dependent PI 3-kinase.

U937:Δp85 cells were primed with IFN-γ 24 hours prior to experiments. To induce Δp85 expression, 15 mM IPTG was added 10 hours prior to experiments. To aggregate FcγRI, cells were loaded with mAb 22 at 4°C followed by goat anti-mouse crosslinking antibody. Control cells were stimulated with goat anti-mouse IgG alone. Cells were warmed to 37°C for the times indicated (minutes) and cell lysates prepared. Western blots of cell lysates (10 μg/sample) were prepared and probed with anti-phospho-ERK antibodies (pERK) or anti-phospho-p38 antibodies (pp38). Blots were stripped and reprobed with pan-ERK (ERK) or pan-p38 (p38) antibodies to ensure comparable loading of cell lysates. Separate blots were also probed with anti-p85 antibodies to check for the expression of Δp85 following IPTG induction (bottom panel). The bands corresponding to endogenous p85 and induced Δp85 are indicated.

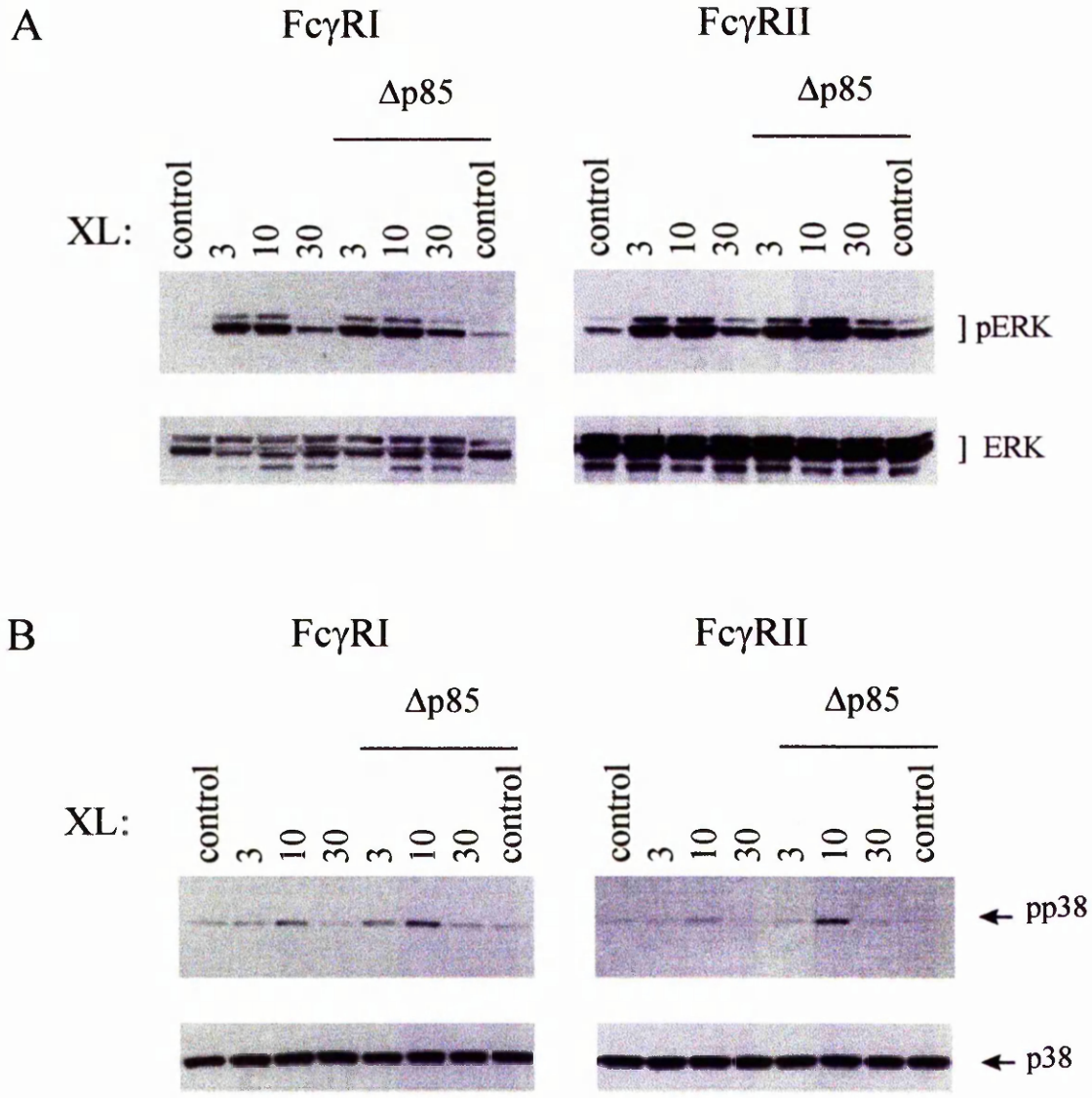
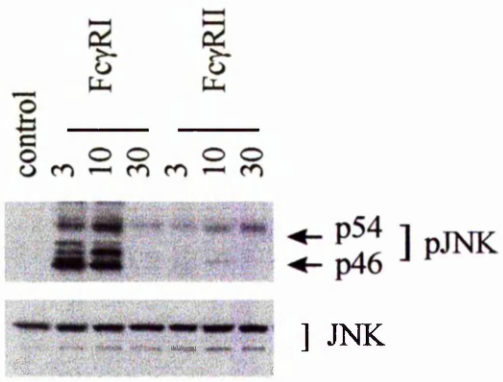

A
FcγRI
FcγRII
Δp85
Δp85
XL:
control
3
10
30
3
10
30
control
control
3
10
30
3
10
30
control
] pERK
] ERK
B
FcγRI
FcγRII
Δp85
Δp85
XL:
control
3
10
30
3
10
30
control
control
3
10
30
3
10
30
control
pp38
p38

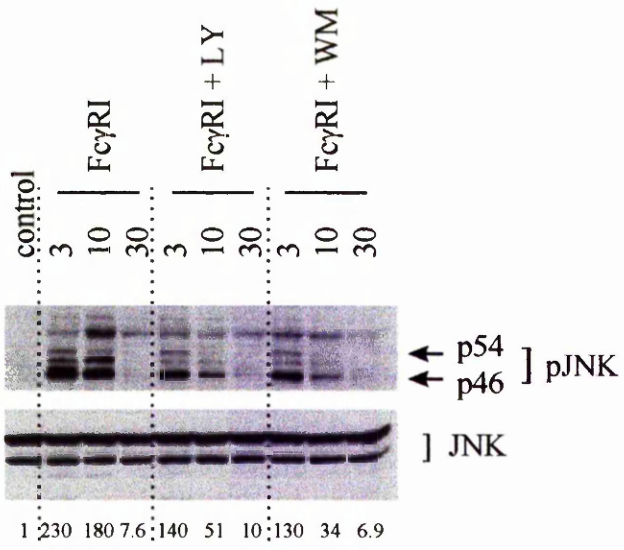
Figure 5.11 FcγRI aggregation activates p46 and p52 JNK.

U937 cells were primed with IFN-γ for 24 hours prior to experiments. (A) Cells were loaded with either anti-FcγRI mAb 22 or anti-FcγRII mAb KB61 at 4°C and receptors aggregated by the addition of goat anti-mouse IgG. Control cells were stimulated with goat anti-mouse IgG alone. Cells were warmed to 37°C for the times indicated. JNK phosphorylation was assessed in cell lysates (10 μg/sample) by Western blot analysis with polyclonal anti-phospho-JNK antibodies (indicated pJNK). Blots were stripped and reprobed with pan-JNK antibodies to control for equal loading (indicated JNK). (B) FcγRI induced JNK phosphorylation is downstream of PI 3-kinase. To inhibit PI 3-kinase cells were incubated for 20 minutes prior to receptor aggregation with either 250μM LY294002 (indicated LY) or 100 nM wortmannin (indicated WM). FcγRI was aggregated as described above. Phospho-JNK and total JNK were detected by Western blot as described above. The numbers beneath these blots indicate the relative intensity of bands as assessed by densitometry, with the control arbitrarily set as 1. These data are corrected for equal loading. (C) Δp85 expression enhances FcγRI induced JNK activation. U937:Δp85 cells were treated as for Figure 5.10. FcγRI was aggregated with mAb 22 for the times indicated and JNK phosphorylation assessed as described above.

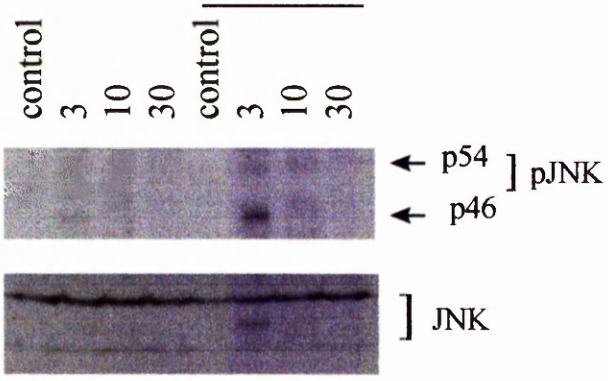
A


control
3
10
30
FcγRI
3
10
30
FcγRII
p54
p46
pJNK
JNK

B


control
3
10
30
FcγRI
3
10
30
FcγRI + LY
3
10
30
FcγRI + WM
p54
p46
pJNK
JNK
1 230 180 7.6 140 51 10 130 34 6.9

C


Δp85
control
3
10
30
control
3
10
30
p54
p46
pJNK
JNK

Chapter 6

Differential recruitment of accessory molecules by FcγRI during monocyte differentiation.

6.1. Introduction

Fc receptors play a pivotal role linking the cellular and humoral arms of the immune response. Each class of immunoglobulin is recognised by a set of receptors; thus, IgG is recognised by Fcγ receptors (FcγRs), IgA by FcαRs, IgE by FcεRs and IgM by FcμRs (Ravetch & Kinet, 1991, Hulett & Hogarth, 1994). The precise distribution of these receptors on cells defines the nature of the cellular response to antigens. On leukocytes, aggregation of Fc receptors by immune complexes leads to a variety of responses such as endocytosis and phagocytosis, activation of the oxidative burst, cytokine release and degranulation. Activation of these receptors can ultimately lead to targeted cell killing through the process of antibody mediated cell cytotoxicity (Fanger *et al.*, 1989), which is critically important for cancer surveillance and the clearance of virus infected cells (Ely *et al.*, 1996).

Three classes of FcγRs have been identified; FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) (section 1.1.3). FcγRI, the human high affinity IgG receptor, comprises an integral type I membrane glycoprotein and is confined in expression to myeloid cells. Aggregation of FcγRI on myeloid cells results in the initiation of tyrosine kinase dependent activation of signal transduction pathways as evidenced by tyrosine phosphorylation and tyrosine kinase dependent cytosolic calcium transients (Liao *et al.*, 1992, Rankin *et al.*, 1993, Davis *et al.*, 1994, Scholl *et al.*, 1992). However, the cytoplasmic tail of FcγRI possesses no known signalling motif. In particular FcγRI lacks an Immunoreceptor Tyrosine Activation Motif (ITAM) (Allen & Seed, 1989). To recruit tyrosine kinases, FcγRI is required to recruit an accessory molecule and this receptor has been shown to associate with the γ-chain (Ernst *et al.*, 1993, Ra *et al.*, 1989, Scholl &

Geha, 1993). The γ-chain, a 7 kDa protein, was first identified as a component of the multisubunit high affinity receptor for IgE, FcεRI (Blank *et al.*, 1989) and belongs to a family of signal transducing molecules which include ζ and η chains (Cambier *et al.*, 1995) of the T cell receptor. These proteins form homo- or hetero-dimers being characterised as integral type I membrane proteins with a short extracellular region, a single transmembrane spanning unit and a cytoplasmic tail containing one or more conserved ITAM motifs (Cambier *et al.*, 1995). The essential role of the γ-chain in linking Fc receptors to intracellular signalling cascades has been demonstrated by the γ-chain knock-out mouse (Clynes *et al.*, 1999).

The FcγRII subfamily of IgG receptors show low affinity for IgG and, thus, these receptors only recognise IgG that has been aggregated by multivalent antigens (section 1.1.3.2). Unlike other IgSF family Fc receptors, FcγRII isoforms contain cytoplasmic signalling motifs. Thus, the cytoplasmic tail of FcγRIIa contains an ITAM motif capable of recruiting tyrosine kinase dependent signalling pathways (Brooks *et al.*, 1989).

U937 cells are a human monoblastic cell line that constitutively express FcγRI and FcγRII but not FcγRIII. These cells also express the IgA receptor, FcαRI. FcαRI, like FcγRI and FceRI, requires the γ-chain for signal transduction (Morton *et al.*, 1995). These cells can be differentiated to a more macrophage phenotype by treatment with dibutyryl cAMP (dbcAMP) (Sheth *et al.*, 1988). We have recently shown that the high affinity receptor for IgG, FcγRI, exhibits a differentiation dependent molecular switch in the nature of the intracellular signalling cascades activated (Melendez *et al.*, 1998a). Thus, in cells primed with IFN-γ, FcγRI activates a novel signalling pathway in which the receptor is coupled to activation of phosphatidyl choline-specific phospholipase D (PC-PLD) (Melendez *et al.*, 1998b). In cells differentiated to a more macrophage-like phenotype with dbcAMP, FcγRI is coupled to the activation of phospholipase C resulting in the generation of inositol 1,4,5 trisphosphate through the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2). The molecular mechanisms regulating this switch were unclear although the coupling appeared to depend on differential recruitment of the γ-chain for signal transduction.

Here, using blocking antibodies, I show that signal transduction in dbcAMP differentiated cells, following the formation of surface immune complexes through ligand binding to FcγRI in dbcAMP differentiated cells has an absolute requirement on recruitment of the low affinity receptor, FcγRII and results in coupling of FcγRI to phospholipase C activation. In these cells, the γ-chain no longer appears to be physically or functionally associated with FcγRI but rather associates with the IgA receptor, FcαRI. In contrast, in cytokine primed cells, signal transduction by FcγRI does not require recruitment of FcγRII as blocking the ligand recognition site has no effect on the phosphotyrosine signal. In these cells, the γ-chain associates with FcγRI not FcαRI. Of interest, recruitment of the γ-chain in either cell type directs coupling of these receptors to PC-PLD activation. Thus, FcγRI in cytokine primed cells and FcαRI in dbcAMP differentiated cells activates PC-PLD.

6.2. Results

6.2.1. FcγRI physically and functionally associates with the γ-chain in IFN-γ primed cells but not in dbcAMP differentiated cells

Immunoprecipitates of FcγRI were Western blotted and probed for the presence of the γ-chain. As expected, the γ chain co-immunoprecipitated with FcγRI in cells primed with IFN-γ (Figure 6.1A). However, in cells differentiated with dbcAMP, the γ-chain could not be detected in FcγRI immunoprecipitates (Figure 6.1A). Following human IgG immune complex activation (section 2.3.1.1), the γ–chain was rapidly tyrosine phosphorylated in IFN-γ primed cells but not in dbcAMP differentiated cells (Figure 6.1B). Taken together, this data indicates that the γ-chain associates physically and functionally with FcγRI in IFN-γ primed cells but not in dbcAMP differentiated cells.

6.2.2. *Activation of signalling cascades by immune complexes requires binding to both FcγRI and FcγRII in dbcAMP differentiated cells but not in IFN-γ treated cells*

The ability of immune complexes to initiate intracellular signalling cascades was measured by Western blot analysis and detection of tyrosine phosphorylated proteins (section 2.3.6.1). Formation of surface immune complexes resulted in the rapid phosphorylation of tyrosine residues on multiple proteins in both IFN-γ primed and dbcAMP differentiated U937 cells (Figure 6.2A). Thus, surface immune complexes are able to initiate signalling cascades in dbcAMP differentiated cells which are independent of γ-chain recruitment. To determine the potential role of FcγRII in mediating the activation of signalling pathways, cells were loaded with the monoclonal antibody IV3 which blocks IgG binding to FcγRII (Kavai *et al.*, 1991). The effect of the presence of mAb IV3 on phosphotyrosine patterns generated by the aggregation of human IgG by surface immune complexes was determined in both IFN-γ primed cells and dbcAMP differentiated cells.

In IFN-γ primed cells, the phosphotyrosine patterns were identical following formation of surface immune complexes in the presence or absence of the anti-FcγRII mAb, IV3. In contrast, loading dbcAMP differentiated cells with IV3 to block FcγRII virtually abolished tyrosine phosphorylation resulting from ligation of FcγRI by surface immune complexes (Figure 6.2A). This observed reduction in the phosphotyrosine profile in the presence of IV3 was not a result of this antibody preventing the binding of human IgG to dbcAMP differentiated U937 cells as FACS analysis demonstrated that binding of fluorescent IgG was unaffected by the presence of IV3 (Figure 6.2B).

Taken together, this data suggests that signal transduction following the formation of surface immune complexes in dbcAMP primed cells requires binding to both FcγRI and FcγRII. In dbcAMP treated cells, binding to FcγRI alone is insufficient to initiate full signalling as evidenced by the deficient profiles of tyrosine phosphorylation observed in the presence of mAb IV3. Thus, this data implies that FcγRI requires FcγRII for signal transduction in dbcAMP differentiated cells. In contrast, in IFN-γ primed cells, FcγRI is able to fully initiate signalling independently of FcγRII. Of interest, in IFN-γ primed cells, the phosphotyrosine patterns obtained following the specific aggregation of FcγRII

using receptor specific monoclonal antibodies differed from that observed for surface immune complexes or anti-FcγRI antibodies (Figure 6.2A). This data together with the ability of FcγRI to associate with the γ chain suggests that, in IFN-γ primed cells, the γ chain accounts for the signalling cascades initiated by FcγRI, following formation of surface immune complexes. In contrast, in dbcAMP differentiated cells, FcγRI no longer uses the γ chain for signal transduction but rather surface immune complexes appear to require the recruitment of FcγRIIa for tyrosine kinase activation.

6.2.3. Expression of the γ-chain is increased by both IFN-γ and dbcAMP differentiation in U937 cells

Next, the reason for the failure of FcγRI to associate the γ-chain was investigated. Firstly, the presence of the γ-chain following differentiation was investigated by Northern and Western blot analysis. Cells were treated with either IFN-γ (200 ng/ml) or dbcAMP (1 mM) and the level of expression of γ chain mRNA was examined at various times. In undifferentiated cells (time 0), only very low levels of the mRNA for γ-chain were detected (Figure 6.3A). Following treatment with IFN-γ, the level of mRNA for the γ-chain was increased above basal at the first time point (6 h) and remained elevated 24 h after treatment with IFN-γ. Treatment of cells with dbcAMP resulted in an increase in the mRNA for the γ-chain such that at 24 h the mRNA showed a 10 fold increase over basal levels.

Changes in γ-chain mRNA expression were mirrored by changes in the protein level as detected by Western blot analysis. Equal amounts of protein (20 μg) from resting cells or from cells treated either IFN-γ or dbcAMP were probed for the presence of the γ-chain (Figure 6.3B). Treatment with IFN-γ for 24 h resulted in a two fold increase in the γ-chain protein. Differentiation with dbcAMP resulted in a larger, 10 fold, increase in the γ-chain expression. This data indicates that the γ-chain is expressed in dbcAMP treated cells; indeed expression is upregulated by dbcAMP. Therefore, the failure of FcγRI to recruit the γ-chain in dbcAMP differentiated cells is not a result of down-regulation of the γ-chain.

6.2.4. FcαRI expression is increased by dbcAMP and in these cells the γ-chain preferentially associates with FcαRI

The γ-chain is known to associate with Fc receptors other than FcγRI; notably, FcαRI, FcεRI, FcγRIII (section 1.1.6). It was hypothesised that, in dbcAMP differentiated cells, the γ chain preferentially associates with another Fc receptor, in particular FcαRI (CD89). Immunoprecipitates of FcαRI were probed for the presence of the γ-chain. In dbcAMP differentiated cells, the γ-chain co-immunoprecipitated with FcαRI. In contrast, in IFN-γ primed cells, only low levels of the γ-chain could be detected in immunoprecipitates of FcαRI (Figure 6.4A). These results contrast to those observed for FcγRI (Figure 6.1A).

Tyrosine phosphorylation patterns were compared following specific aggregation of FcαRI using anti-CD89 monoclonal antibodies in IFN-γ primed or dbcAMP differentiated cells. Aggregation of FcαRI in dbcAMP differentiated cells resulted in rapid tyrosine phosphorylation of multiple proteins whereas the signal was minimal following aggregation in IFN-γ primed cells (Figure 6.4B). Specific tyrosine phosphorylation of the γ-chain was investigated in the two cell states following aggregation of FcαRI. Thus, in dbcAMP differentiated cells, the γ-chain was tyrosine phosphorylated following FcαRI aggregation using CD89 specific antibodies whereas, in IFN-γ primed cells, a similar approach resulted in a very small phosphotyrosine signal despite equal loading of γ-chain immunoprecipitates (Figure 6.4C).

The relative expressions of FcγRI and FcαRI in IFN-γ and dbcAMP treated cells were measured using Northern blot (section 2.5.2) and FACS analysis (section 2.2.2.1). FcγRI is known to be tightly regulated by IFN-γ in U937 cells and, consistent with this, IFN-γ resulted in a large transient increase in the mRNA for FcγRI (Figure 6.5A) and this was matched by an increase in the mean fluorescent intensity of the cell surface receptor (Figure 6.5B). In contrast, the mRNA for FcαRI was reduced in cells treated with IFN-γ although the mean fluorescent intensity of the cell surface FcαRI remained essentially unchanged by IFN-γ treatment (Figures 6.5A and 6.5B).

In cells differentiated with dbcAMP, the opposite effects were observed. Thus, FcγRI expression was reduced whereas FcαRI expression was increased (Figure 6.5A). After 12 h exposure to dbcAMP, the mRNA levels for FcαRI had increased 8 fold above basal and then gradually declined over time such that by 48 h levels had returned approximately to basal levels. For FcγRI, 24 h after dbcAMP differentiation, the mRNA was no longer visible by Northern blot analysis (Figure 6.5A). A significant increase in mean surface fluorescence for FcαRI was observed after 48 h differentiation with dbcAMP (Figure 6.5B).

Thus, differentiation with dbcAMP results in the upregulation of expression of both FcαRI and γ-chain and in these cells, the γ-chain co-immunoprecipitates with FcαRI but not FcγRI. Furthermore, specific aggregation of FcαRI results in enhanced tyrosine phosphorylation of the γ-chain in dbcAMP differentiated cells when compared to IFN-γ primed cells. Taken together, this data indicates that in IFN-γ treated cells, the γ-chain is predominantly associated with FcγRI whereas in dbcAMP differentiated cells, the γ-chain is associated with FcαRI.

6.2.5. FcαRI is coupled to Phospholipase D activation in dbcAMP differentiated cells

In IFN-γ treated cells, FcγRI couples to the activation of PC-PLD via the γ-chain (16). Conversely, in cells differentiated with dbcAMP, FcγRI no longer associates with the γ chain or couples to the activation of PC-PLD. This data implies that receptor coupling to PC-PLD requires the γ-chain. As FcαRI associates with the γ-chain in dbcAMP treated cells, the ability of this receptor to activate PC-PLD was investigated.

The coupling of γ-chain to PC-PLD activation was confirmed (section 2.4.1) (Figure 6.6). In IFN-γ treated cells, specific aggregation of FcαRI did not lead to a significant increase in PC-PLD activity (Figure 6.6). However, in dbcAMP differentiated cells, specific aggregation of FcαRI resulted in an increase in PC-PLD activity (Figure 6.6). Thus, in dbcAMP differentiated cells, FcαRI is coupled to the activation of PC-PLD in a γ-chain dependent manner.

6.3. Discussion

Here I have demonstrated that differentiation of U937 cells alters the nature of the Fc receptor that associates with the γ-chain and changes the activation mechanism of cells by IgG immune complexes. Both IFN-γ and dbcAMP increase expression of the γ-chain. However, the γ-chain associates physically and functionally with FcγRI only in IFN-γ primed cells. In dbcAMP differentiated cells, the γ-chain no longer associates with FcγRI but rather functionally couples FcαRI to intracellular signalling cascades. In both IFN-γ and dbcAMP differentiated cells, γ-chain recruitment results specifically in activation of PC-PLD. In the absence of functional coupling of FcγRI with the γ-chain in dbcAMP differentiated cells, IgG containing immune complexes initiate an alternative signalling pathway through the recruitment of FcγRII to FcγRI.

The cytoplasmic tail of FcγRI contains no known signalling motif (Allen & Seed, 1989) and a functional association with the γ chain is well-recognised (Ernst *et al.*, 1993, Ra *et al.*, 1989, Scholl & Geha, 1993). Here, I show that the γ-chain associates with FcγRI only in IFN-γ primed cells. Using identical immunoprecipitation conditions, the γ-chain in dbcAMP differentiated cells did not associate with FcγRI. We have previously shown that dbcAMP differentiation results in a switch in the nature of the signalling pathways activated by surface immune complexes (Melendez *et al.*, 1998a). Differential recruitment of the γ-chain was proposed as the mechanism underlying this switch although the mechanism regulating this switch was unclear. The failure of FcγRI to recruit the γ-chain cannot be explained by its absence in dbcAMP differentiated cells as here we have shown that expression of the γ-chain is upregulated following differentiation. It appears rather that following differentiation, the γ-chain preferentially associates with FcαRI.

Two possible reasons may account for the switch in Fc receptor association of the γ-chain. The first lies in the predicted nature of the interaction of the two chains as this appears to be more robust for interactions between FcαRI and the γ-chain than for the

other receptors. Thus, association of γ-chain with FcαRI can be observed in cell membranes solubilised with 1% Nonidet P-40 whereas the same detergent disrupts the association of γ-chain with FcγRI (Launay *et al.*, 1999). The association between γ-chain and FcγRI can only be observed in the presence of mild detergents such as 1% digitonin (Launay *et al.*, 1999). Recruitment of the γ-chain is dependent on the transmembrane domains of FcαRI and FcγRI (Lang, Shen & Wade, 1999, Launay *et al.*, 1998). FcαRI possesses an arginine residue in its transmembrane domain and this residue is essential for the recruitment of the γ-chain presumably through the formation of an ion pair interaction with the aspartate of the γ-chain. Mutation of the arginine to leucine resulted in failure to recruit the γ-chain and thereby trigger the signalling cascades (Lang *et al.*, 1999, Pfefferkorn & Yeaman, 1994). In contrast, the mechanism of interaction of the γ-chain with FcγRI is unknown. Although the transmembrane domain of FcγRI is necessary and sufficient for the functional recruitment of the γ-chain (Launay *et al.*, 1999, Morton *et al.*, 1995), there is no positively charged amino acid in the predicted transmembrane domain of FcγRI (Allen & Seed, 1989). A similar ion pair interaction between the juxtamembrane histidine residue of FcγRI with the aspartate of the γ-chain has been proposed (Lang *et al.*, 1999) but shown not required for functional γ-chain recruitment (Davis *et al.*, 1995b).

The second possible explanation for the differential Fc receptor association with the γ-chain following differentiation is that the two proteins associate during their synthesis and passage through the endoplasmic reticulum. Both IFN-γ and dbcAMP treatment increase mRNA and protein expression of the γ-chain. However, IFN-γ differentially increased expression of FcγRI but downregulated FcαRI. In these cells the γ-chain is predominantly associated with FcγRI. In contrast, dbcAMP upregulated expression of FcαRI and downregulated FcγRI and, in these cells, the γ-chain appears to be predominantly associated with the FcαRI. A possible explanation for the findings here are that, during nascent synthesis, the γ-chain in the endoplasmic reticulum associates with the corresponding nascent Fc receptor and that this complex, once formed is stable and excludes the other Fc receptor once it reaches the plasma membrane.

Although FcγRI no longer associates with the γ chain in dbcAMP differentiated cells, surface immune complexes are still able to trigger intracellular signalling cascades as

evidenced by the tyrosine phosphorylation. In dbcAMP differentiated cells, the low affinity receptor, FcγRII, is upregulated and this receptor possesses an unusual ITAM and is able to couple efficiently to tyrosine kinase activation (Cambier *et al.*, 1995). Here I show that surface immune complexes require binding to both FcγRI and FcγRII to initiate signalling. One possibility is that in these cells, FcγRI forms heterodimeric complexes with FcγRII and this is required to induce efficient signal transduction.

The role of IgA and IgG in regulating myeloid cells is complex. Previous studies have shown that the two receptors co-operate on peripheral blood mononuclear cells and enhance phagocytosis release (Fanger, Goldstine & Shen, 1983, Shen & Fanger, 1981). However, serum IgA (monomeric) has also been shown to be anti-inflammatory and capable of inhibiting IgG mediated phagocytosis, oxidative burst and cytokine (Wolf *et al.*, 1994, Wolf *et al.*, 1996, Nikolova & Russell, 1995). The differential levels of expression of the two receptors and their mutually exclusive coupling to the γ-chain during differentiation may in part account for these different responses.

Figure 6.1 FcγRI couples to γ-chain in IFN-γ primed U937 cells but not in dbcAMP differentiated cells.

(A) FcγRI was immunoprecipitated from U937 cells treated with IFN-γ for 24 hours or dbcAMP for 48 hours. Immunoprecipitates were subjected to SDS-PAGE, blotted to nitrocellulose and probed with anti-γ-chain antibodies. (B) U937 cells were either primed with IFN-γ or differentiated with dbcAMP as indicated. FcγRI was aggregated by incubation with either human IgG followed by goat anti-human IgG. Control cells were stimulated with goat IgG alone (control). Cells were warmed to 37°C for 1 min, lysed and subjected to immunoprecipitation with anti-γ-chain antibodies. Immune precipitates were subjected to SDS-PAGE, blotted to nitrocellulose and probed with 4G10 anti-phosphotyrosine antibody (top panel). Blots were stripped and reprobed with anti-γ-chain antibody (bottom panel).

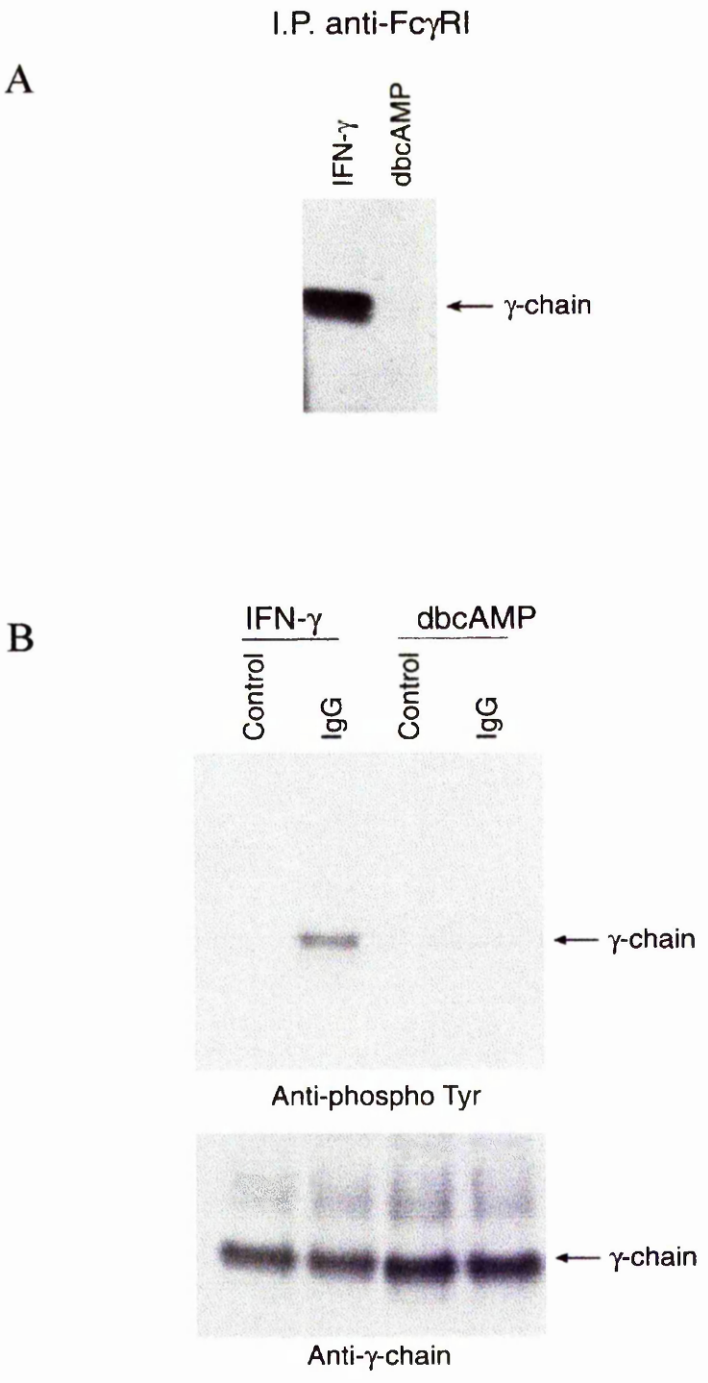
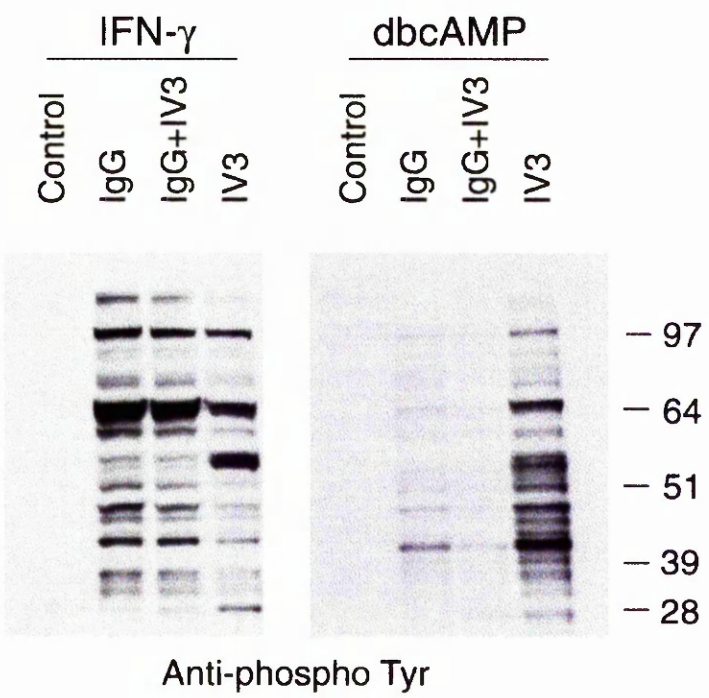

I.P. anti-FcγRI
A
IFN-γ
dbcAMP
γ-chain
B
IFN-γ
dbcAMP
Control
IgG
Control
IgG
γ-chain
Anti-phospho Tyr
γ-chain
Anti-γ-chain

Figure 6.2 FcγRI mediated activation of signalling cascdes in dbcAMP differentiated cells, but not in IFN-γ primed cells, requires FcγRII.
(A) IFN-γ and dbcAMP treated cells were stimulated by the formation of surface immune complexes with human IgG, followed by goat anti-human IgG. The effect of blocking the ligand binding pocket of FcγRIIa was ascertainned by preincubating the cells with the mAb IV3 prior to formation of surface immune complexes (IgG + IV3). For comparison, cells were also stimulaed by aggregation of FcγRIIa with mAb IV3 followed by anti-mouse IgG (IV3). Control cells were stimulated with goat IgG alone (control). (B) The effect of the FcγRIIa blocking antibody, IV3, on IgG binding was examined by FACS analysis. Cells differentiated with dbcAMP were loaded with IgG:FITC in the presence or absence of mAb IV3 as indicated. Cells with no IgG:FITC were included to control for autofluorescence (*). Cells were analysed as outlined in section 2.2.2.2.

A


IFN-γ
dbcAMP
Control
IgG
IgG+IV3
IV3
Control
IgG
IgG+IV3
IV3
97
64
51
39
28
Anti-phospho Tyr

B

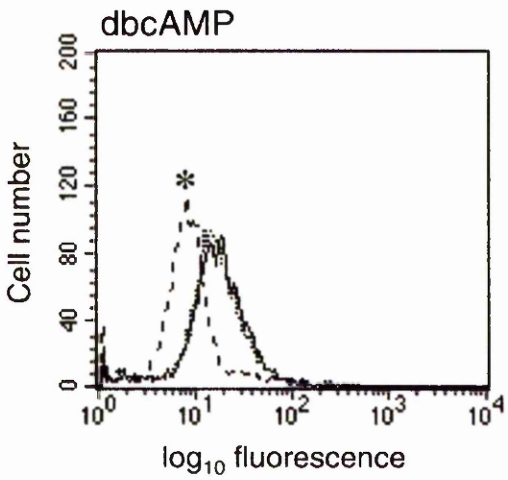
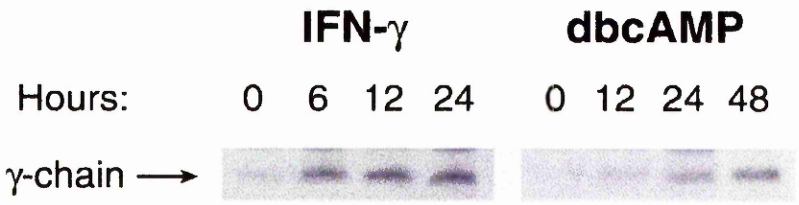

dbcAMP
Cell number
0
40
80
120
160
200
*
10^0
10^1
10^2
10^3
10^4
$\log_{10}$ fluorescence

Figure 6.3 Expression of γ-chain is increased by IFN-γ and dbcAMP differentiation.

(A) Northern blot analysis of γ-chain mRNA expression. U937 cells were treated with IFN-γ or dbcAMP and cells harvested at the times indicated. Total RNA (20 μg/sample) was subjected to Northern blot analysis as described in materials and methods. Blots were hybridised with a ^{32}P-labelled γ-chain cDNA probe. (B) Western blot analysis of γ-chain protein levels. Equal quantities of protein (20 μg/sample) from untreated U937 cells (control), cells treated with IFN-γ for 24 hours and cells differentiated with dbcAMP for 48 hours were subjected to Western blot analysis as indicated in materials and methods. Blots were probed with rabbit anti-γ-chain antibody.

A


IFN-γ
dbcAMP
Hours:
0 6 12 24
0 12 24 48
γ-chain

B

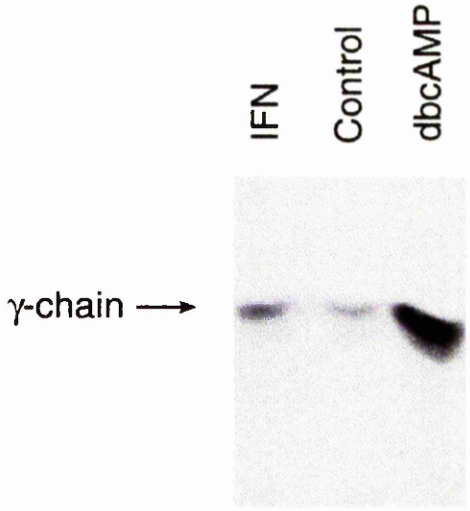
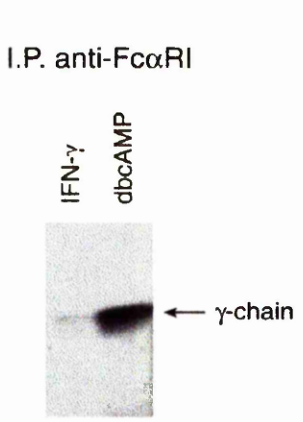

IFN
Control
dbcAMP
γ-chain

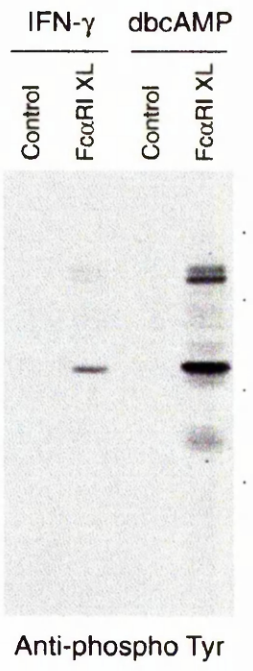
Figure 6.4 In cells differentiated with dbcAMP, γ-chain preferentially associates with FcαRI.

(A) FcαRI was immunoprecipitated from U937 cells treated with IFN-γ for 24 hours or dbcAMP for 48 hours. Immunoprecipitates were Western blotted and probed for γ-chain. (B) Aggregation of FcαRI with mAbs induces the tyrosine phosphorylation of multiple proteins.U937 cells were either primed with IFN-γ or differentiated with dbcAMP as indicated. FcαRI was aggregated with the FcαRI specific mAb A3 followed by goat anti-mouse IgG for 1 min at 37°C. Control cells were stimulated with goat anti-mouse IgG alone (control). Tyrosine phosphorylation in cell lysates (20 μg/sample) was assessed by Western blotting with the 4G10 anti-phosphotyrosine antibody (bottom panel). (C) FcαRI aggregation induces the phosphorylation of γ chain. Cells were stimulated as in (B). Following lysis, γ-chain was precipitated from equivalent amounts of cell lysate (500 μg). Immunoprecipitates were subjected to SDS-PAGE, blotted to nitrocellulose and probed with 4G10 anti-phosphotyrosine antibody (top panel). Blots were stripped and reprobed with anti γ-chain antibody (bottom panel).

A


I.P. anti-FcαRI
IFN-γ
dbcAMP
γ-chain

B


IFN-γ
dbcAMP
Control
FcαRI XL
Control
FcαRI XL
Anti-phospho Tyr

C

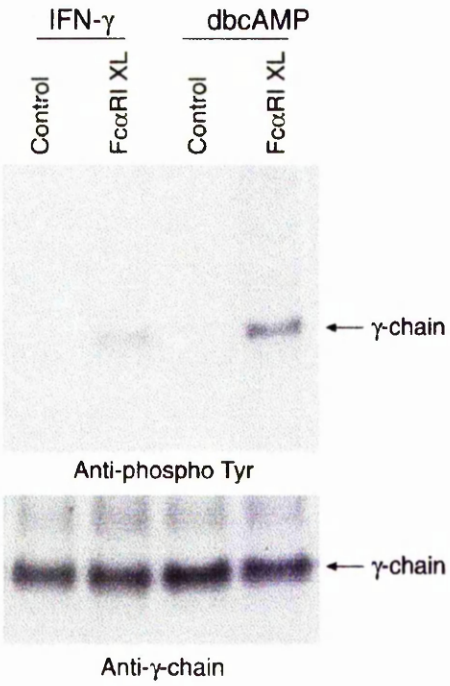
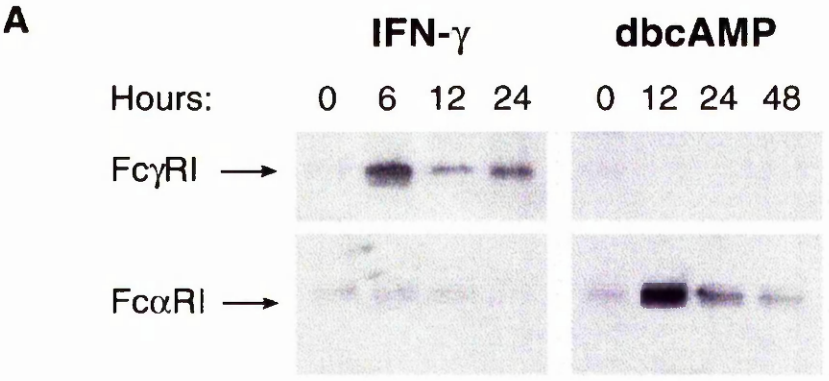

IFN-γ
dbcAMP
Control
FcαRI XL
Control
FcαRI XL
γ-chain
Anti-phospho Tyr
γ-chain
Anti-γ-chain

Figure 6.5 Differentiation with dbcAMP or priming with IFN-γ alters relative expression of FcγRI and FcαRI

(A) Northern blot analysis of FcγRI and FcαRI expression. Cells were treated with either IFN-γ or dbcAMP and harvested at times shown (hours) for isolation of RNA. Total RNA (20 μg/sample) was subjected to Northern blot analysis as described in materials and methods. Blots were hybridised with specific ^{32}P-labelled cDNA probes for either FcγRI or FcαRI as indicated. (B) FACS analysis of FcγRI and FcαRI surface expression. Untreated U937 cells and cells treated with either IFN-γ (24 hours) or dbcAMP (48 hours) were labelled with either the FcγRI specific mAb 22 (left panel), or FcαRI specific mAb A3 (right panel). An irrelevant mouse IgG1 was used as an isotype control (*). A goat anti-mouse IgG:FITC was used as a secondary antibody and cells were subjected to FACS analysis as described in section 2.2.2.1.


A
IFN-γ
dbcAMP
Hours:
0 6 12 24
0 12 24 48
FcγRI
FcαRI

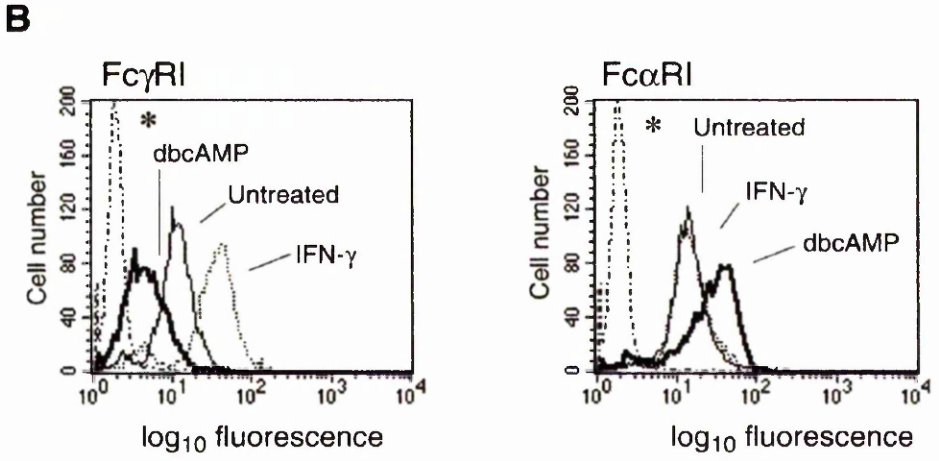
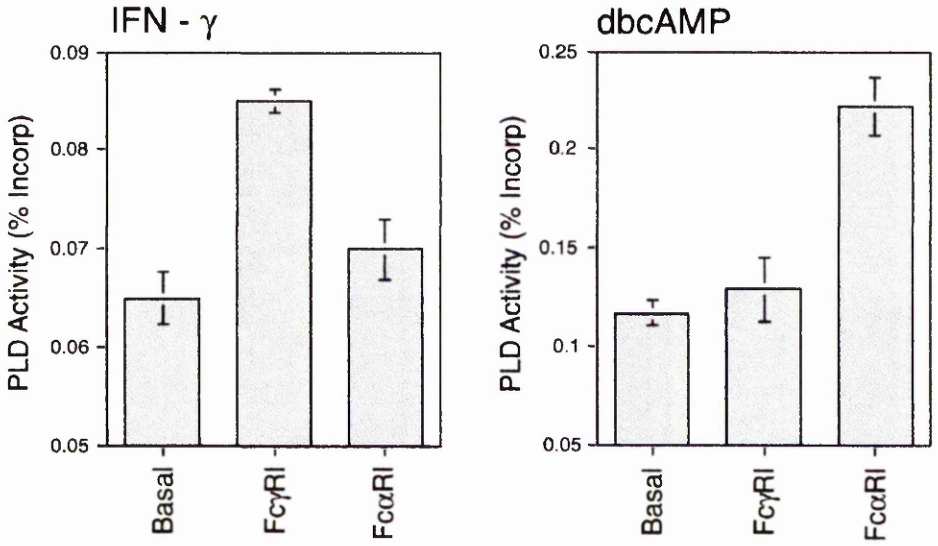

B
FcγRI
FcαRI
Cell number
log10 fluorescence
dbcAMP
Untreated
IFN-γ
*

Figure 6.6 Coupling of receptors to the γ-chain correlates with activation of PC-PLD.

PLD activity following crosslinking of FcγRI or FcαRI with specific mAbs was measured using the PLD-transphosphatidylation assay (section 2.4.1). U937 cells were treated with IFN-γ or dbcAMP, labelled overnight with [^{3}H] palmitic acid and preincubated with butan-1-ol for 30 min prior to the assay. FcγRI and FcαRI were labelled with mAb 22 and mAb A3 respectively. The accumulation of [^{3}H]PtdBut in cells over 30 min following the addition of goat anti-mouse crosslinking antibody was measured. Control cells were incubated with crosslinking antibody but no mAb (control).


IFN - γ
PLD Activity (% Incorp)
0.09
0.08
0.07
0.06
0.05
Basal
FcγRI
FcαRI
dbcAMP
PLD Activity (% Incorp)
0.25
0.2
0.15
0.1
0.05
Basal
FcγRI
FcαRI

Chapter 7

General Discussion

7.1. Fcγ receptors mediate critical immune functions

Fcγ receptors expressed on the surface of monocytes and macrophages mediate a number of critical immune functions. These include the internalisation of immune complexes by endocytosis and phagocytosis, activation of the respiratory burst, release of cytokines and ADCC (van de Winkel & Capel, 1993, Ravetch, 1994, Daeron, 1997a). The signalling pathways linking Fcγ receptors to these responses however have proved difficult to define. This is due, at least in part, to marked heterogeneity in signalling pathways activated by immune complexes in primary monocytes and macrophages. To overcome these difficulties it is necessary to use more defined model systems, such as cell lines, to study Fcγ receptor signal transduction.

7.2 Fcγ receptor signalling in U937 cells depends on the differentiation state of the cell

In this study, I have used the U937 cell line as a model for immune complex signalling in monocytes and macrophages. U937 cells undergo controlled differentiation when treated with dbcAMP into a more macrophage-like cell (Sheth *et al.*, 1988). Alternatively, U937 cells can be primed to behave like activated monocytes by treatment with IFN-γ (Harris & Ralph, 1985). Using this system, previous work has shown that the signal transduction pathway activated by immune complexes fundamentally depends on the differentiation state of the cell (Melendez *et al.*, 1998a). Thus, in dbcAMP differentiated macrophage-like cells, Fcγ receptor aggregation induces the activation of PLCγ1, prolonged $InsP_3$ production and sustained calcium signalling (Floto *et al.*, 1997, Davis *et al.*, 1995a). Conversely PLC is not activated in monocyte like IFN-γ primed U937 cells. In these cells, Fcγ receptor aggregation activates a novel signal transduction pathway involving the sequential activation of PC-PLD and sphingosine kinase, resulting in a single

transient cytosolic calcium spike (Melendez *et al.*, 1998b, Floto, Mahaut-Smith & Allen, 1995). Further, the lack of PLC activation and calcium influx in response to Fcγ receptor aggregation in IFN-γ primed cells is not simply a result of differentiation dependent changes, as specific aggregation of the low affinity IgG receptor, FcγRIIa, with monoclonal Abs in the same cells results in both PLCγ1 activation and also prolonged calcium signalling (Melendez *et al.*, 1998a). In this thesis I have addressed a number of potential mechanisms which might underlie this developmental switch in signalling pathway activated by FcγRI.

7.3. What governs the nature of the calcium transients generated by Fcγ receptors in U937 cells?

Calcium signalling plays a central role in activation by many immune receptors in all immune cells studied. In these cells, calcium signalling is biphasic. Following receptor ligation, calcium is released from internal stores, resulting in a transient rise in cytosolic calcium. This, in turn, opens store operated calcium channels in the plasma membrane, termed I_{CRAC}, leading to prolonged elevation of calcium or calcium oscillations (Hoth & Penner, 1992). The ER Ca^{++} ATPase inhibitor, thapsigargin, depletes internal calcium stores and results in a rise in cytosolic calcium (Thastrup *et al.*, 1990). As treatment of cells with thapsigargin results in the opening of Icrac calcium channels it has long been assumed that calcium release from internal stores was sufficient to trigger for calcium influx. As previous work has demonstrated that aggregation of FcγRI in IFN-γ stimulated U937 cells results in release of calcium stores, but no calcium influx, I set out to investigate possible inhibitory mechanisms which might prevent the opening of I_{CRAC}.

Looking to other immune receptors for clues, it was noted that the calcium transients generated by FcγRI signalling in IFN-γ primed cells closely resembled calcium transients seen in response to ligation of the B-cell receptor (BCR) under conditions of 'negative' signalling. Aggregation of the BCR under positive signalling conditions, results in the activation of PLCγ2, $InsP_3$ production and prolonged calcium influx, resulting from both store release and I_{CRAC} opening. However, co-ligation of the BCR with the inhibitory low affinity receptor for IgG, FcγRIIb, results in release of calcium from internal stores but no

calcium influx through I_{CRAC} (discussed in section 1.2.8.9). Here, the 5' inositol phosphatase, SHIP, is recruited to FcγRIIb where it hydrolyses the major PI 3-kinase product, $PI(3,4,5)P_3$ (PIP_3) to $PI(3,4)P_2$. As PIP_3 is required for PLCγ activation and the prolonged $InsP_3$ production required to initiate calcium influx in these cells, SHIP provides a selective block in calcium influx while leaving many other tyrosine kinase dependent events intact (Scharenberg & Kinet, 1998, Sarkar *et al.*, 1996, Scharenberg & Kinet, 1996). In summary, BCR co-ligation with FcγRIIb involves the activation of tyrosine kinases, low PLCγ activation, low $InsP_3$ production, calcium release from internal stores and no calcium influx. The close identity of this signalling phenotype with the phenotype induced by FcγRI in IFNγ primed U937s made this an attractive potential mechanism for investigation.

Thus, two parallel approaches were taken to investigate a possible role for the 5'inositol phosphatase, SHIP, in the regulation of FcγRI signalling. Firstly, a role for SHIP was analysed directly by immune precipitation studies and biochemical assays (see Chapter 3). Secondly, as the low affinity receptor for IgG, FcγRIIb is the only receptor definitively proven to recruit SHIP '*in vivo*', I set out to investigate whether this receptor was expressed in U937 cells (see Chapter 4).

In chapter 3 I demonstrate that, SHIP does appear to play a role in FcγRI signalling in IFN-γ primed U937s. Thus, following FcγRI aggregation SHIP is rapidly tyrosine phosphorylated and associates with the adapter protein Shc. Further, Shc translocates to the plasma membrane and both SHIP and Shc appear to be recruited to immune complexes. This was proposed as a potential mechanism to explain the lack of PLCγ activation and calcium influx observed for FcγRI signalling (Cameron & Allen, 1999).

A number of points don't however tie up with this hypothesis. Firstly, PI 3-kinase activation induced by FcγRI is robust with prolonged PIP_3 production detectable following receptor ligation (Melendez *et al.*, 1998c). Secondly, no FcγRIIb expression was detectable in these IFN-γ primed cells (see Chapter 4) and no other ITIM bearing receptor has been reported to associate with FcγRI. Finally, SHIP-Shc interactions were also found to occur in response to FcγRII aggregation, which induces both $InsP_3$

production and calcium influx. Consistent with a non-inhibitory role for SHIP-Shc interactions, Ingham *et al* recently demonstrated that SHIP expression is required for the correct phosphorylation of Shc (Ingham *et al.*, 1999). As Shc phosphorylation has been implicated in the activation of the MAP kinase cascades (Harmer & DeFranco, 1997), this role for SHIP appears to be independent of the inhibitory actions mediated by FcγRIIb recruitment of SHIP in B-cells.

To our surprise, FcγRIIb expression was found in dbcAMP differentiated cells where Fcγ receptors couple to PLCγ activation and calcium influx (Chapter 4) (Davis *et al.*, 1995a, Melendez *et al.*, 1998a). The role of FcγRIIb in these cells remains elusive, though some preliminary data suggests it might temper the activation of specific signalling events, such as PKB, activated by FcγRIIa. This is the subject of ongoing investigation.

In IFN-γ primed U937 cells, FcγRI mobilises calcium from intracellular stores via an $InsP_3$ independent mechanism. Here, the sequential activation of PLD and sphingosine kinase leads to calcium release presumably through the SCaMPER calcium channel which is found in the endoplasmic reticulum (section 1.4.3) (Mao *et al.*, 1996, Melendez *et al.*, 1998b). Why does this $InsP_3$ independent pathway to Ca^{++} store release not lead to calcium influx through I_{CRAC}? Parekh *et al* have extensively studied $InsP_3$ dependent calcium mobilisation in rat bosophilic leukemia cells and their findings provide a possible explanation for our observations (Parekh & Penner, 1997, Parekh, Fleig & Penner, 1997). They have demonstrated in single cells that intracellular calcium store release and the activation of I_{CRAC} have very different sensitivities to $InsP_3$ (Parekh *et al.*, 1997). Thus, significant calcium store release occurs at $InsP_3$ concentrations as low 60-100 nM while Icrac is only activated at μM concentrations of $InsP_3$. This allows dissociation of calcium store release and calcium influx as is observed during FcγRIIb mediated inhibition of calcium entry. Parekh *et al* hypothesise that this difference in sensitivity results from functionally distinct calcium stores, one resulting in a transient rise in cytosolic calcium and the other resulting in I_{CRAC} opening. Due to the high concentrations of $InsP_3$ required to activate I_{CRAC} they further suggest that this second, less $InsP_3$ sensitive, calcium store is likely to lie close to the plasma membrane where local $InsP_3$ concentrations will be highest (Parekh & Penner, 1997, Parekh *et al.*, 1997). If two independent calcium stores

do indeed exist it is possible that the novel sphingosine kinase dependent calcium mobilisation pathway activated by FcγRI in IFN-γ primed cells is unable to mobilise the stores required to operate I_{CRAC}. Thus the failure of FcγRI to induce calcium influx through I_{CRAC} may depend, not on the termination of PIP_3 dependent responses by SHIP, but rather on the failure of FcγRI to couple to PLCγ activation.

7.4. What governs the switch in phospholipase pathway activated by Fcγ receptors?

In chapter 5, the reason for the failure of FcγRI to couple to PLCγ activation was investigated. Both FcγRI and FcγRII couple to the activation of Lyn and Syk tyrosine kinases and patterns of tyrosine phosphorylation generated in response to aggregation of either receptor are very similar (Agarwal *et al.*, 1993, Kiener *et al.*, 1993). Further, both FcγRI and FcγRII induce the phosphorylation of PLCγ1 (Liao *et al.*, 1992). However, it has been demonstrated that tyrosine phosphorylation of PLCγ, though essential for activation, does not necessarily correlate with activity (Rhee & Bae, 1997). Thus, under some circumstances PLCγ can be heavily phosphorylated while showing low activity. Recently, the mechanism governing PLCγ activation by various immune receptors has been elucidated. PLCγ activation is now known to require phosphorylation by both Syk and Tec family tyrosine kinases (section 1.2.4.3). Thus, in the case of FcεRI, activation of PLCγ1 is mediated by Syk and Btk (a Tec family kinase). Further, a number of adapter proteins have been shown to coordinate the recruitment and phosphorylation of PLCγ by these kinases. In FcεRI signalling, the membrane associated adapter, LAT, anchors PLCγ1 at the plasma membrane while the adapter protein SLP-76 coordinates the phosphorylation of PLCγ1 by Btk (section 1.2.4.3). To investigate whether differences in PLCγ1 activation mediated by FcγRI and FcγRII in IFN-γ primed U937 cells resulted from differences in the recruitment and correct phosphorylation of PLCγ1, adapter protein complexes were examined. In chapter 5, I demonstrate that FcγRII appears to directly recruit constitutive SLP-76:PLCγ1 complexes to the plasma membrane. No interaction of SLP-76 or PLCγ1 with the γ-chain, the signalling subunit of FcγRI, could be detected. This provides a potential mechanism for the lack of PLC activity observed for FcγRI aggregation in IFN-γ primed cells.

In dbcAMP differentiated cells, aggregation of either FcγRI or FcγRII results in PLCγ activation. Conversely, neither receptor couples to the activation of PC-PLD in these cells (Melendez *et al.*, 1998a). As FcγRII is upregulated in dbcAMP differentiated cells and as FcγRII aggregation induces PLC activation in IFN-γ primed cells, it was hypothesised that FcγRI might couple to PLCγ activation by recruiting FcγRII. Further, it was hypothesised that activation of PLD by FcγRI in IFN-γ primed cells was dependent solely on a γ-chain mediated signal. Consistent with this hypothesis anti-sense oligonucleotides against FcγRIIa were found to reduce the coupling of FcγRI to PLC in dbcAMP differentiated cells while anti-sense oligonucleotides to γ-chain had no effect. Conversely, in IFN-γ primed cells, anti-sense oligonucleotides to γ-chain reduce FcγRI coupling to PLD, while FcγRIIa anti-sense had no effect (Melendez *et al.*, 1998a). The reason for the failure of FcγRI to couple to PLD in dbcAMP differentiated cells was not clear.

In chapter 6, data is presented which explains both the coupling of FcγRI to PLC in dbcAMP treated cells and also explains the failure of FcγRI to couple to the activation of PLD. Using the mAb, IV3, which blocks IgG binding to FcγRIIa (Kavai *et al.*, 1991), we demonstrated that, in dbcAMP differentiated cells, initiation of tyrosine phosphorylation by immune complex was dependent on the recruitment of FcγRIIa (Figure 7.1). In IFN-γ primed cells this was not the case as FcγRIIa blocking antibodies had no effect on tyrosine phosphorylation. I further demonstrated that association of FcγRI with the γ-chain correlates with the ability of the receptor to activate PC-PLD. Thus in IFN-γ treated cells, FcγRI physically and functionally associates with high levels of γ-chain and activates PC-PLD. Conversely, in dbcAMP differentiated cells, no γ-chain could be detected in association with FcγRI providing an explanation for the failure of FcγRI to activate PLD in these cells. As dbcAMP differentiated cells express very high levels of γ-chain, it was also not clear why the γ-chain failed to associate with FcγRI in these cells. However, as other receptors also associate with the γ-chain, it was hypothesised that in dbcAMP cells, γ-chain might preferentially associate with another receptor. Consistent with this argument I found that the IgA receptor, FcαRI, was upregulated in these cells

and was associated with the γ-chain. Further, I found that FcαRI activated PC-PLD in these cells, consistent with the γ-chain specifying activation of this pathway (Figure 7.1).

These data taken together indicate that the signalling pathway activated by Fcγ receptors depends entirely on the signalling chain used to initiate signal transduction. Thus, the γ-chain specifies a PC-PLD pathway while FcγRIIa specifies PLCγ1. Further, the ability of FcγRI to use these signalling chains in U937 cells is dependent on the maturation state of the cells. Both γ-chain and FcγRIIa initiate signal transduction by recruiting tyrosine kinase to ITAM motifs within their cytoplasmic tails. Interestingly, FcγRIIa bears an atypical ITAM with 14 amino acids separating the two key tyrosine residues as opposed to the 10 amino acid separation seen for the γ-chain (section 1.2.1.1). It is tempting to speculate that this difference is directly responsible for the switch in phospholipase pathway activated.

It is still not clear, however, why FcγRI exclusively couples to PLD activation in IFN-γ primed cells when the data from dbcAMP differentiated cells clearly demonstrates that FcγRI can recruit FcγRIIa to activate PLCγ1. It is possible that FcγRI association with the γ-chain might exclude FcγRIIa from signalling complexes, perhaps by inducing the dimerisation of FcγRI.

Recently, a dependence of phagocytosis on the activation of PLD has been proposed (Kusner *et al.*, 1996, Kusner *et al.*, 1999, Lennartz, 1999). It is perhaps surprising, therefore, that we observe no PC-PLD activation following specific ligation of FcγRIIa, as this receptor is capable of supporting phagocytosis. Some reports have indicated that FcγRIIa, like other Fc receptors, is capable of functional interaction with the γ-chain (Masuda & Roos, 1993). This is, however, still the subject of some debate and we have found no evidence for such a mechanism in our system. Alternatively, FcγRIIa might activate forms of PLD with different substrate specificity, such as the reported cytosolic PLD which preferentially hydrolyses phosphatidylinositol or phosphatidylethanolamine (Pertile *et al.*, 1995). This possibility is currently under investigation.

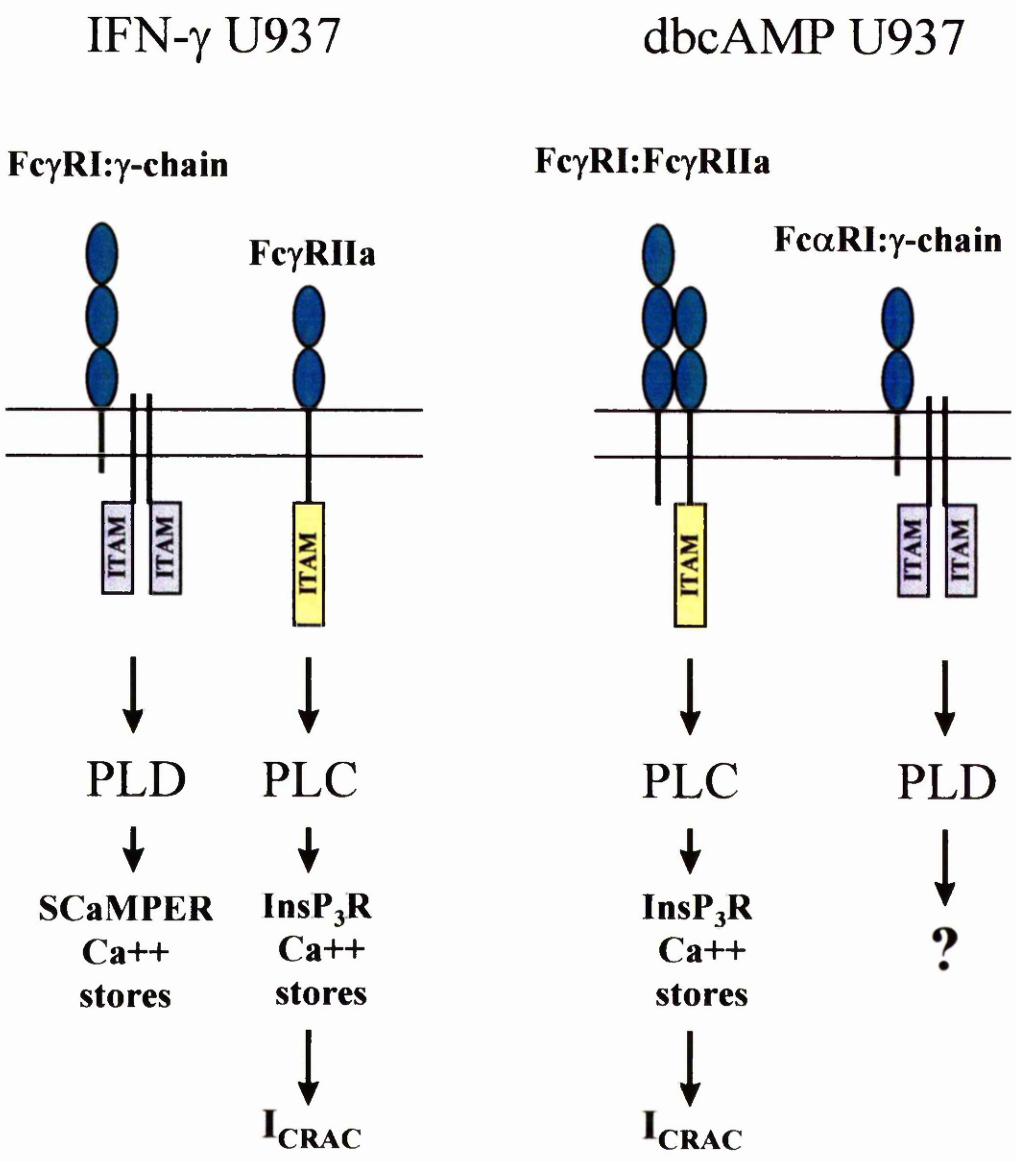


Figure 7.1 Model for differentiation dependent switch in signalling pathway activated by FcγRI.

7.5. The functional consequence of two independent Fcγ receptor signalling pathways

Interestingly, in murine systems, Fcγ receptors rely entirely on the γ-chain for tyrosine kinase mediated signal transduction, as a counterpart to FcγRIIa does not exist in mouse. This has been conclusively demonstrated by generation of γ-chain deficient mice (Clynes *et al.*, 1998, Clynes *et al.*, 1999). Interestingly, mice which lack the γ-chain show protection from immune complex mediated tissue damage and also show less susceptibility to auto-immune disease in a number of models. The reason for the existence of two functionally distinct ITAMs for Fcγ receptor signalling in human systems is not entirely clear. Some clues, however, are provided by the expression patterns of the different Fcγ receptors in human systems (section 1.1.3). Thus, FcγRIIa is expressed on neutrophils, monocytes and macrophages where it appears to act as a major pathway for immune complex clearance. Interestingly, FcγRIIa has also been identified as the major receptor for C-reactive protein (CRP), indicating a role for this receptor in acute inflammatory responses and innate immunity (Bharadwaj *et al.*, 1999). FcγRI conversely is more restricted in expression, being tightly controlled by IFN-γ. FcγRIIIa, which also associates with the γ-chain is found expressed on macrophages and also on NK cells (section 1.1.3.3). It has been shown that FcγRI, FcγRIII and FcαRI but not FcγRIIa, can support ADCC implying a dependence on the γ-chain ITAM for this process (section 1.1.8.3). As ADCC is a process capable of inducing tissue injury, separation of this effector function to a separate signalling subunit might provide a level of protection against incorrect activation of this process. It has also been suggested that FcγRI, but not FcγRIIa can support antigen presentation (Van den Herik-Oudijk *et al.*, 1995b). These studies were however carried out in A20 murine B-cell transfectants so much caution must be taken when interpreting these data, particularly as murine systems entirely lack FcγRIIa.

Further to a switch in the phospholipase pathway, I have shown here, for the first time, that FcγRI and FcγRII couple to distinct patterns of MAP kinase activation in IFN-γ primed cells. Thus, FcγRI aggregation activates ERK, p38 and JNK MAP kinase cascades, while FcγRIIa activates only ERK and p38. Further, FcγRI and FcγRII appear

to couple to the MAP kinase cascades via alternative pathways. This is discussed in detail in Chapter 5. When taken together with differences in the calcium transients it seems likely that the two receptors will couple to distinct transcriptional responses. Future work will attempt to identify both transcription factors and transcriptional targets regulated by these receptors, with particular reference to the production of inflammatory mediators. These data perhaps imply that γ-chain mediated and FcγRIIa mediated immune complex signal transduction might play distinct roles in the regulation of inflammation by mediating distinct transcriptional responses.

Intriguingly, different Fc receptors differ in their affinities for Ig classes and IgG subclasses (section 1.1.3.4). This implies that the response of monocytes and macrophages to immune complexes depends not only on the repertoire of Fc receptors expressed, but also on the composition of the immune complex. This is most strikingly demonstrated for IgG2 immune complexes as only FcγRIIa is capable of binding IgG2. At the other extreme, IgG4 immune complexes do not appear to bind FcγRIIa with any measurable affinity but these complexes will bind FcγRI and the inhibitory receptor, FcγRIIb (van de Winkel & Capel, 1993). As different Ig classes and IgG subclasses are associated with different types of immune response, this is likely to be of importance in immune system regulation.

In summary, two independent IgG immune complex signalling systems appear to exist in human leukocytes, one dependent on the γ-chain and the other dependent on FcγRIIa. In U937 cells, the high affinity receptor for IgG, FcγRI is capable of interacting with both of these signalling subunits and this interaction is dependent on the differentiation state of the cell. Further, the signal transduction pathways activated by the γ-chain and FcγRIIa in U937 cells are extremely different. By simply altering levels of expression of the different Fcγ receptors, along with levels of associated γ-chain, human monocytes and macrophages can vary both their affinity for IgG complexes and their functional responses to these immune complexes. This versatility is likely to account for much of the heterogeneity of responses observed in primary monocyte and macrophage systems.

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Publications

The following publications arose from work associated with this thesis.

Cameron AJ, Harnett MM, Allen JM. Differentiation dependent switch in the coupling of Fc receptors to phospholipase D in U937 cells. Meeting Abstract. Immunology, 1999, Vol 98: 19.4, p59.

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