Studies on Mechanisms of Fcγ Receptor Internalisation of Antigen

By

BRYONY CLAIRE MELVILLE

A Thesis presented for the degree of Masters

in

Faculty of Medicine and Therapeutics at the University of Glasgow

Davidson Building
Glasgow G12 8QQ

August 1998

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Abstract

A differential pattern of tyrosine kinase phosphorylation for distinct FcγR signalling domains was defined in several ways. Firstly, Syk recruitment was observed in interferon gamma (IFNγ) and dibutyl cAMP (DBC) treated U937 monocyte-like cells, upregulating FcγRI + γ chain of the FcεRI and FcγRII receptors respectively. IFNγ cells showed peak Syk activation at 5-7 minutes post-activation, while DBC cells show an immediate increase in Syk recruitment. In addition, blots of post-activation lysates of the two cell types were probed with anti-phosphotyrosine antibody, revealing a strong band of 110kDa on both IFNγ and DBC blots, and an unknown 50kDa band found exclusively on the DBC blot. Lastly, experiments designed to test whether or not the two cells types display alternative NO_2 release patterns post-activation were established.

In a second approach, a chimeric receptor was made by fusion of the extracellular region of FcγRI to the transmembrane and cytoplasmic tail of zeta chain. This chimeric receptor was then compared to other similarly constructed mutants, FcγRI-γ and FcγRI-II for its ability to phagocytose sheep red blood cells (SRBC) in a well established phagocytosis experiment using COS-7 cells (a simian kidney fibroblast cell line). All mutants demonstrated relatively equivalent phagocytic ability in the COS-7 cell system. Select tyrosine kinases were then cotransfected with each mutant FcγR receptor. There was no marked difference in distinct tyrosine kinases hFyn, mLyn, and mSyk required by distinct internal tyrosine kinase activation motifs (ITAMs) for increasing phagocytic efficiency. However, it was discovered that the effect of the tyrosine kinase mSyk on FcγRI/γ, FcγRI/ζ, and FcγRI/II internalisation was very significant, dramatically increasing both the number of COS-7 cells capable of phagocytosis and the number of SRBCs phagocytosed by each individual COS-7 cell.

Table of contents

Chapter 1

Introduction

1.1 Human Fc Receptors

The primary functions of macrophages are to phagocytose and destroy foreign micro-organisms and present MHC complexed antigen fragments on their cell surface to helper T-cells. These essential humoral and cellular immunity functions are both mediated in macrophages and in other cell types by the specialised recognition receptors, FcR, which bind the constant region of immunoglobulins. Each immunoglobulin class has a specific Fc receptor that recognises it, i.e. FcαR binds IgA, FcεR binds IgE, FcμR binds IgM, and FcγR binds IgG **(Hulett and Hogarth, 1994; van de Winkel, 1996)**. When a subclass of antibody surrounds antigen present in an organism, the distinctive constant region of the eclipsing antibody is bound by its constant region to specific receptors on cells. When bound to immune cells, an important immunological response is initiated. In this thesis primarily the IgG receptor FcγR and its role in host defence was investigated. The FcεR receptor, whose activation stimulates the IgE-mediated allergic response, will be mentioned for comparisons of critical function and association with signalling subunits **(Ravetch and Kinet, 1991)**.

1.2 Effector Functions of Fcγ Receptors

The Fc region of antibody/antigen complexes bind Fcγ receptors on hematopoietic cells. Subsequently these receptors aggregate and a wide range of downstream responses can be elicited, including ADCC (antigen dependent cellular cytotoxicity), secretion of mediators, and internalisation of particles either by endocytosis (of small immune complexes) or by phagocytosis (of opsonised antibody-coated particles > than 1 mm). Receptor cross-linking is the crucial preceding step to these immunological responses (with the exception of endocytosis through specific receptors discussed in a later section)**(Lin *et al* 1994)**. Cellular activation induced through each of the Fcγ receptors triggers similar biological functions in specific cells (Table 1.1).

Table 1.1 Expression profile of Fc receptors in hematopoietic cells. Table taken from Ravetch and Kinet, 1991.

FcR Family

	Macrophage	Monocyte	Neutrophil	NK cell	B cell	T cell	Mast cell	Basophil
hFcγRI	+	+	+i	-	-	-	-	-
mFcγRI	+	?	?	-	-	-	-	-
hFcγRIIA	+	+	+	-	-	-	?	?
hFcγRIIB	+	+	-	-	+	-	?	?
hFcγRIIC	+	+	+	-	-	-?	?	?
mFcγRII	+	+	+	-	+	+	+	?
hFcγRIIIAα	+	+i	-	+	-	+	?	?
hFcγRIIIB	-	-	+	-	-	-	?	?
mFcγRIIIα	+i	?	+	+	-	-	+	?
hFcεRIα	-	-	-	-	-	-	+	+
mFcεRIα	-	-	-	-	-	-	+	+

i = inducible

FcγR stimulation on macrophages in particular also induces tyrosine kinase (TK) activation, ADCC, cytosolic free Ca^{2+} elevation, release of inflammatory inhibitors, and internalisation of opsonised particles. Macrophages produce vasodilators like nitric oxide that allow migration of immune cells to areas of infection. They also produce proteases which degrade the intracellular matrix of ingested antigen. Activated macrophages act as a cytotoxic agent by harnessing O_2 to form (ROI)-super oxides H_2O_2 and OH groups for so called 'respiratory burst,' a more effective killing of phagocytosed organisms **(Segal & Abo, 1993)**. The degree of FcγR cross-linking correlates to the amount of superoxide produced in activated cells **(Ravetch & Kinet, 1991)**. Degraded antigen bit-sequences can be subsequently expressed on the surface of macrophages in association with MHC class II molecules in the classic Antigen (Ag) Presentation Model. This mechanism permits recognition by the $CD4^+$ TCR, stimulating T-helper cell activation in areas of infection.

Other soluble mediators produced by macrophages include: leukotrienes, prostaglandins, hydrolytic enzymes and multiple cytokines; IFNγ, IL-1, IL-6, and TNFα **(van de Winkel *et al*, 1991)**. It is a distinct possibility that the nature of the receptor mediating antigen entry may determine the cellular response. The individual contribution of specific receptor classes is only starting to be elucidated.

1.3 Structure of IgG Fc Receptors

Of the Ig-related Fc receptor family involved in cell signalling processes, structurally different FcR extracellular domains (EC), transmembrane domains (TM) and intracellular domains (IC) may ultimately be linked to alternative signalling pathways being initiated in distinctive cell types.

At the moment the subclass of IgG binding hFc receptors has three major classes of leukocyte FcγR; FcγRI, II, and III. Although multiple mRNA transcripts and genes for the different Fc receptors exist, the cDNA of all three classes are similar, predicting structurally equivalent proteins. Indeed most FcR's are integral type I glycoproteins having a leader peptide, an amino terminus extracellular portion with 2-3 Ig-like ligand binding domains, a single pass transmembrane domain, and a carboxy terminal cytoplasmic tail(41). The specific FcγR's are distinctive due to variations in molecular size, affinity and specificity for IgG, cellular distribution, and recognition by monoclonal antibodies **(Hulett and Hogarth, 1994)**.

A critical exception to the Ig superfamily related FcγR's is the FcγIIIb proteins, translated as an integral membrane type I protein, but cleaved to a glycosyl phosphatidyl-inositol GPI-anchor protein as a result of post-translational modifications. Although the extracellular domains function like classic FcγRIIIa proteins, the FcγIIIb lacks membrane spanning and cytoplasmic regions, cordoning it for easy removal from cellular membranes and acting as soluble Fc receptors channelled through the organism. There is some evidence to support a functional immunological role for these soluble Fc Receptors **(van de Winkel & Capel, 1993)**.

1.3.1 FcγRI (CD64): Nomenclature, Isotypes, Structure, and Ligand Binding

FcγRI is encoded from 3 genes, hFcγRIA, B, C located on chromosome 1 band 1p12-13 and 1q21.1 **(Ernst et al., 1992; Allen & Seed, 1989)**. Each RI gene is

composed of 6 exons. Two exons encode the signal peptide, 3 exons encode one each of the (Ig)-superfamily like extracellular domains, and the last exon encodes in combination the TM and IC regions **(van de Winkel & Capel, 1993)**. Each gene encodes a single transcription product apart from hFcγRIB gene which encodes two, hFcγRIb1 and b2, making a total of 4 transcription products.

FcγRIa is a 72 kDa glycoprotein, or a 55 kDa core protein (non-glycosylated) **(Hulett & Hogarth, 1994)**. It is the only receptor class to bind monomeric IgG with high affinity (K_D $3x10^8$ M^{-1}) equilibrium affinity constant (Ka), of 10^8-10^9 M^{-1} by Scatchard analysis **(Allen & Seed, 1989)**. FcγRIa, will be from now on referred to as FcγRI. FcγRI shows subtype IgG specificity of IgG3>IgG1>IgG4>>>IgG2.

The extracellular domain of FcγRI has 3 Ig-like domains(292aa), a hydrophobic transmembrane region(21aa), and a highly charged cytoplasmic domain(61aa) **(van de Winkel *et al*, 1993)**. FcγRI contains no recognised signalling motif, so it associates primarily with the γ-chain of the FcεRI high affinity receptor. Through this γ chain's intrinsic ITAM, signal transduction events are initiated inside the cell **(Küster *et al* 1990; Ernst *et al* 1993; Scholl and Geha 1993)**.

1.3.2 FcγRII (CD32): Nomenclature, Isotypes, Structure, and Ligand Binding

hFcγRII is encoded by three genes, A, B, and C located on chromosome 1 band 23-24 **(van de Winkel *et al.*, 1993)**. IIA and IIB isotypes differ in signal peptide and IC domains. IIC resembles IIA in the IC domain, and IIB in the signal peptide domain. It is likely that gene C results from an unequal cross-over event between genes A and B. While IIA and IIC are the most similar, all three types have the same overall intron and exon organisation. Gene IIC is actually a pseudogene because a point mutation renders it a translation termination codon.

The three FcγRII genes consist of 6 exons; 2 exons for signal peptides, 1 exon for each of 2 Ig-like domains, 1 exon for the TM region and 1 for the IC region. Alternative splicing gives rise to 6 transcripts FcγRIIa1, a2, b1, b2, b3, c **(van de Winkel and Capel 1993; Hulett and Hogarth 1994)**. Of the 6 transcripts 5 show low affinity for IgG (Ka<107M-1). The FcγRIIa2 transcript has an alternative TM splicing rendering it a soluble receptor protein.

The glycosylated form of hFcγRIIa1 has Mr of 40 kDa, while the core protein has a Mr of 36kDa. The extracellular domain of FcγRIa1 has 2 Ig-like domains(180aa), a TM region(27-29aa), and most interestingly an IC domain(76 aa), with a single intrinsic ITAM signalling motif (Internal Tyrosine-kinase Activation Motif). FcγRIIa1 has only a low affinity for monomeric IgG ($K_D < 10^7 M^{-1}$), and much more readily binds complexed IgG **(van de Winkel and Capel 1993)**. There are two allelic forms of the FcγRIIa1 receptor, each binding IgG subtypes with non-equivalent affinities. The high responding allele FcγRIIa1(HR) binds IgG1=3>>2,4 , while the low responding allele FcγRIIa1(LR) binds IgG1,2,3>>4 **(Hulett & Hogarth, 1994)**. The FcγRIIa1 transcript is the most common type II receptor and will from now on be referred to as FcγRII.

1.3.3 FcγRIII (CD 16): Nomenclature, Isotypes, Structure, and Ligand Binding

The hFcγRIII receptor is encoded from 2 distinct genes A & B **(Ravetch & Perussia, 1989)**. The type B gene is identical to the type A gene apart from a single aa change from Phe-203 in type A to a Ser-203 which is responsible for a termination codon (CGA to UGA) intrinsic to the sequence for type B. The Phe in the A form maintains an integral membrane glycoprotein crucial for preservation of TM and cytoplasmic domains. The B form, in contrast, is GPI anchored to the outer plasma membrane leaflet.

The FcγRIIIa has an Mr between 50-80KDa seeing as it precipitates as a broad band. It has 2 Ig-like domains of 190aa, a transmembrane region with a 25 amino acid cytoplasmic tail. FcγRIII also has only a low affinity for monomeric IgG ($K_D < 10^7 M^{-1}$) **(Ravetch & Kinet, 1991)**.

FcγRIIIA receptor has been shown to associate with the γ-chain subunit of the FcεRI in macrophages and also the ζ-chain of TCR in NK cells. These subunits are responsible for the signalling ability of cells stimulated through the FcγRIIIa, from now on referred to as FcγRIII. FcγRIIIB is unable to mediate cell killing.

1.4 Sequence Homology Between Receptor Classes

Interestingly the TM sequence within FcγRI and III receptor groups demonstrate high sequence homology (50-76%). This is in line with the belief that the TM regions of FcγRI and III are responsible for subunit recruitment.

There is relatively low homology of the TM of the FcγRII, precisely because it does not depend on these sequences to recruit effector proteins. Also FcγRI and FcγRIII exhibit low sequence homology of the cytoplasmic region between each distinctive group. Only FcγRII, which signals through its own IC tail maintains high sequence homology between isotypes (about 60%) **(Hulett & Hogarth, 1994)**.

All three classes of human FcγR genes are found located on the long arm of chromosome 1 **(Hulett & Hogarth, 1994)**. However, in the mouse genome high affinity Fcγ receptors (1q22) are found on chromosome 1 while the low affinity Fcγ receptors (band 1q12-13, and 1q21) are found on chromosome 3 **(Osman *et al.*, 1992)**. This has preempted many scientists in the field to believe the FcγR's share convergent evolutionary ties, or at least linkages diverged much farther back than previously believed **(Ravetch, 1994)**.

1.5 Expression Of Fcγ Receptors

There is differential expression of the IgG Fc receptor isoforms on different cell types. The cell type in which a particular IgG receptor is expressed determines the biological response elicited. Some of the factors involved are the substrates available in different cell types and the strength and persistence of the stimuli needed to elicit a response. The signalling cascade may be linked to unique functioning capabilities of the cell, say nitric oxide production in macrophages and histamine release in granulocytes. However, researchers are uncovering different receptor isoforms in the same cell type that can recruit specific substrates and elicit distinct responses.

1.5.1 Expression of FcγRI

FcγRI is expressed on macrophages (10^5), monocytes (4×10^3), and myeloid cells. Expression on macrophages and monocytes is cytokine modulated, for example PMN can be induced by interferon-γ (IFNγ) and (IL-10) to express this receptor, but normally it is not found in high numbers at the cell surface **(Guyre *et al.*, 1989)**. Monocytes stimulated with IFNγ have FcγRI upregulated 4-5 fold (60,0000 receptors per cell). Further, an IFN-γ responsive element has been found on the promoters of both FcγRIA and IB genes **(Pearse *et al.*, 1991)**. It is the high affinity receptor for monomeric IgG that is over-

expressed on monocytes in infected areas and this is the most affective receptor at antigen internalisation.

1.5.2 Expression of FcγRII

FcγRII is the most widely expressed receptor of the three and is found on all types of blood leukocyte cells apart from NK cells. On macrophages there are $8x10^4$ receptors while on platelets there are about 1,500. It appears that FcγRII is the only FcγR type on Langerhans cells and on placental endothelial and trophoblast cells **(van de Winkel and Capel, 1993)**. IIA and IIC isotypes are only found in neutrophils while IIB is only type found in B or T lymphocytes. FcγRIIb1 and 2 have a 13 amino acid intracellular sequence, EAENTITYSLLKH, essential for endocytosis, antigen presentation, and inhibition of B cell activation. However, an inhibitory sequence insert that hampers clathrin coated pit localisation in the b1 isoform makes it unable to mediate antigen presentation. B cells express the FcγRIIb1 isotype. Therefore B cells are unable to effectively present immune complexes, except for rheumatoid factors.

Of the alternative splicing receptors of the FcγRIIB gene, the IIb1 class is expressed exclusively on mature lymphocytes and while the IIb2 class is expressed on early myeloid cells, suggesting a function for their alternative cytoplasmic domains **(van de Winkel and Capel, 1993)**.

Although there has been no evidence of cytokine inducement of FcγRII receptor upregulation on distinct cell types, U937 cells can be induced using dibutyl cAmp into a more macrophage cell type, resulting in an increase in FcγRII receptor type expressed on the cell surface. It is believed that a similarly simulated differentiation may occur in vivo during monocyte differentiation.

1.5.3 Expression of FcγRIII

FcγRIII is found primarily on NK cells and is present in small amounts on macrophages, T-cells, and (10%) on monocytes. It is the sole IgG receptor class to be expressed in mesangial kidney cells, and placental trophoblasts, and on NK cells. Since ADCC is a characteristic of activated NK cells, perhaps FcγRIII is specialised for this function. In contrast FcγRIIIB-GPI anchor form is found mainly on neutrophils and eosinophils as serum receptors in cleaved form. Its expression is cytokine regulated with G-CSF,

GM-CSF, IFN-γ, and TNF-β showing the ability to upregulate expression while TNF-α and IL-4 show the ability to inhibit or downregulate expression **(Hulett & Hogarth, 1994)**. Although FcγRIIIb expression is restricted in neutrophils and eosinophils, it has been found that normal serum contains high levels of FcγRIIIb receptor, probably originating from neutrophils, and released by some proteolytic method or perhaps generated by alternative splicing. Soluble forms of FcγR's may be involved in the regulation of IgG production **(Kinet, 1989)**.

1.6 Disease Relevance of Polymorphisms

FcγRI polymorphisms are few, however, there is a family in Belgium that has absent FcγRI on all of their cells, even after donated samples of their monocytes and neutrophils were incubated with IFNγ, an inducer of FcγRI. Interestingly, these individuals appeared to be disease free, so seemingly other Fc receptors compensate for the absent FcγRI **(Ceuppens *et al* ., 1988)**.

Interestingly, Prins and group in 1993 discovered that the non-obese diabetic mouse had a 73 aa deletion and a 17 aa substitution in the FcγRI receptor cytoplasmic tail. They showed a reduced ability of this receptor to endocytose immune complexes, linking the variant FcγRI receptor allele to type 1 diabetes in these mice.

In contrast FcγRII has a distinctive polymorphic product termed HR versus LR. This polymorphism is recognised by MAb 41H.16 which binds to an epitope of the high responder allele(HR) while not the low responder allele(LR). 70% of Caucasian individuals are HR and can be induced by mIgG1 anti-CD3 mAb to initiate T-cell mitogenesis. Only 30% of Caucasians are LR. Asians have reversed distribution, with 15% HR and 85% LR **(Tax *et al* 1983; Abo *et al* 1984; Warmerdam *et al* 1990; Hulett and Hogarth 1994)**. The two polymorphisms HR versus LR differ by 2 aa, a His to Gln switch at position 27 and a Arg to His switch at position 131 respectively. Since IgG2 isotype specific bacterial antigens are known, individuals with the $Fc\gamma RII^{HR}$ may be more protected against these disease agents than $Fc\gamma RII^{LR}$ individuals. Indeed Burgess and group in 1995 found that $Fc\gamma RII^{LR}$ individuals were predisposed to heparin-induced thrombocytopenia (HIT). The heparin-dependent antibody is predominantly of the IgG2 subclass. Platelet aggregation mediated through the FcγRII on these cells is hampered when $Fc\gamma RII^{LR}$ does not recognise the IgG2 associated herapin complex.

The FcγRIIIB(GPI) form has a NA1/NA2 polymorphism. NA1/NA1 individuals have a FcγRIII protein molecular weight of 29kDa and while NA2 /NA2 individuals have a protein molecular weight of 33kDa due to aa changes creating extra glycosylation spots. Relevance of this has not been elucidated.

Both sFcγRII and sFcγRIII exist although the sFcγRIII is the predominant form. Recombinant sFcγRII inhibited B cell production of anti-SRBC antibodies in mice in a dose dependent fashion (**Fridman *et al.*, 1992**). Only the extracellular domains of the FcγR's were necessary for this action. Also sFcγR's were 10 times more concentrated in samples of serum from IgG secreting tumours of diseased BALB/c mice compared to controls (**Fridman *et al.*, 1992**). This finding implicate sFcγR's in regulation of B cell antibody production. When IgG binds membrane FcγR's in large concentrations, increased cellular activation may initiate increased sFcγR production and secretion, which acts in a circulatory fashion to downregulate IgG antibody production in B cells (**Fridman *et al.*, 1987**).

Interestingly, patients with paroxysmal nocturnal hemoglobinuria disease have FcγRIIIB lacking the GPI anchor tail on their neutrophils. Perhaps FcγRIIIb deficient people are predisposed to this disease condition.

The state of glycosylation of the IgG molecule's constant region has been found to have disease relevance in its binding capacity to Fc receptors. In patients with arthritis a state of glycosylation found in minor concentration in IgG of normal patients was present in extreme amounts in these patients. This altered glycosylation state was found in all four types of circulating IgG's. This suggests that the glycosylation pattern of IgG may regulate adaptive responses in Fc receptors, even if in arthritis it triggers immune cells to inflame otherwise normal tissues (**Dwek *et al.*, 1995**). There is an overproduction of IgG4 in chronic synovial joints of patients with rheumatoid arthritis. Fc binding reactivity on mast cells may contribute to the disproportionate inflammation and mediators released from these cells in joints of patients with this condition (**Zack *et al.*, 1995**).

1.7 Subunits

FcγRIIA has a cytoplasmic domain that contains an intrinsic motif allowing it to signal Ca^{2+} flux and initiate phagocytosis in the absence of recruited signalling subunits (**Odin *et al.*, 1991**)(**Ernst *et al.*, 1993**). In contrast, the human FcγRI and FcγRIII receptors both lack intrinsic signalling motifs. Both

FcγRI and FcγRIII has been shown capable of associating with disulphide-linked dimers of the γ chain (7 kDa) of the FcεRI high affinity receptor and the ζ chain (16 kDa) of the T cell receptor **(Letourneur *et al.*, 1991)(Ernst *et al.*, 1993)(Daeron *et al.*, 1994)**. It has been suggested that FcγRI may also interact and signal through its recruitment of FcγRII.

As most of the research conducted on subunit signalling has involved its association with receptor complexes other than FcγR's, I will give a brief description of FcεRI and TCR, as they are often the models used for ζ chain and γ chain study respectively. I will also mention the natural killer cell, for the human zeta subunit is also long been known to associate with the FcγRIIIA receptor on these cells **(Weissman *et al.*, 1988a & b)**.

1.7.1 FcεRI Structure and Associated γ Chain

The high affinity receptor for IgE responsible for allergic responses is found exclusively on the surfaces of mast cells and basophils cells. It is a tetrameric protein $\alpha\beta\gamma_2$ composed of one IgE binding single pass protein α subunit, resembling two Ig-like domains of the FcγR's, a 4 pass β subunit, and a homodimer of disulphide-linked gamma chains **(Blank *et al.*, 1989)**. The γ chain has one signalling motif with 7 variable amino acids between the two YXXL boxes(X7).

1.7.2 The TCR and Associated ζ, η and γ Chains

The TCR is an oligomeric complex composed of 6-7 subunits **(Klausner *et al.*, 1991)**. The α/β antigen binding subunit is found on most mature T cells. The CD3 complex exists as 3 proteins encoded from homologous clustering genes that couple in the following way; two glycoproteins γ and δ and 1 nonglycosylated protein ε. There are two dimers of CD3 components per receptor, containing one copy of ε and one copy of either δ or γ. The last components are covalent dimers of the signalling ζ family of molecules. In Cytotoxic T cell lines three different dimer types have been found in TCR , ζ–ζ, ζ–η, η–η. Heterodimer ζ and η association with γ of the FcεRI has also been reported **(Orloff *et al.*, 1990)**.

The ζ chain is a nonglycosylated protein of 16 kD while the related η chain of 22 kDa results from an alternative splicing event of the ζ gene **(Frank *et al.*, 1990)**. The zeta chain has a short 9 residue extracellular domain, a

single hydrophobic transmembrane spanning domain and a 113 amino acid tail in the cytoplasm **(Weissman *et al.*, 1988a & b)**.

1.7.3 NK Cells and Associated ζ and γ Subunits

Natural killer cells are large granular lymphocytes that mediate cytotoxicity against tumours and virus-infected cells in a manner not restricted by the major histocompatibility complex, MHC. Perturbation of the the low affinity FcγRIII receptor, the only FcγR on NK cells, triggers its cytolytic activity. It has been shown that FcγRIII on natural killer cells specifically associates with the CD3 ζ homodimer originally found co-associating with the T-cell receptor **(Weissman *et al.*, 1988)(Lanier *et al.*, 1989)** . Indeed the γ–γ chain, γ–ζ chain, and ζ–ζ chain are all expressed at the NK cell surface and are involved in FcγRIII signalling **(Anderson *et al.*, 1990a)**. While dimers η–η, η–ζ, and η–γ have been found to associate with the TCR, so far there is no evidence that the η chain interacts with FcγR's and is expressed on cells other than T cells **(Ravetch & Kinet, 1991)**.

1.7.4 Structural Relativeness of ζ -family subunits: ζ, γ, and η

Both γ and ζ/η chains are located on chromosome 1 **(Ravetch, 1994)**. It has been suggested that an original FcR subunit, through duplications, recombinations, and diversifications was the predecessor of all related FcR subunits recognised today **(Kuster *et al*, 1990)**.

The first two exons in both γ and ζ genes encode a leader sequence, a short extracellular sequence, a TM sequence and the start of the IC domain. γ exons 3-5 and ζ exons 3-7 encode the rest of the cytoplasmic tail. The first three IC exons in both γ and ζ (3-5) have 50% homology **(Ravetch & Kinet, 1991)**.

Lastly, all three members of the zeta family have conserved cysteine residues. The γ chain has 2 N-terminal Cys residues, and ζ and η chains have one. In both γ and ζ/η these residues come together to form disulphide linked dimers. In this form they associate with the Fc receptors **(Ravetch & Kinet, 1991)**. There is an aspartic residue in common with the TM domains of γ and ζ/η. It is believed that this strangely charged aa in the hydrophobic region of the TM domain is involved in FcγR binding. The three major classes of Fc receptors and their associated subunits are shown in Figure 1.1.

Although there is considerable homology between the ζ,γ and η subunits, a main and critical difference is the variations in their signalling motifs. The varying motifs may recruit unique effector molecules that may regulate alternative signalling events in distinctive cell types. Along the same lines of thinking, the structural relatedness of the ζ, γ and η signalling subunits and the capability of heterologous dimers to form between groups has allowed speculation that heterologous coupling may allow for a variety of signalling responses to ensue after activation of the specific receptor. In contrast, homologous coupling of distinct subunits may be a way of achieving unique motif-mediated biochemical responses **(Frank *et al.*, 1990) (Orloff *et al.*, 1990)**. The extent to which this is true is still under investigation.

1.8 Receptor-Subunit Interactions

The FcεRI and FcγRIII share a high homology region in their TM domains (LFAVDTGL), where the negatively charged aspartic D is conserved. This negatively charged residue is likely to be crucial in functional interactions with critical residues of ζ-chain family of dimers. Indeed, FcεRI and FcγRIII receptors that fail to interact with signalling subunits are degraded in the ER and never reach the membrane surface after expression **(Ernst *et al.*, 1993)**. For example, co-expression of the FcγRIII with the human ζ or γ chain permits membrane expression of the complex in COS-7 cells **(Lanier *et al.*, 1989)(Ravetch, 1989)**. Likewise, co-expression of the γ-homodimer with the high affinity IgE Fc receptor present on mast cells and basophils allows for surface expression **(Miller et al., 1990)**.

In conjunction with this, although not involving Fc receptors, the absence of the ζ chain has been described as the limiting factor in complete surface expression of the TCR complex. Indeed, for the α or β subunits of the TCR to be expressed in COS-7 cells, the zeta chain must be simultaneously cotransfected **(Miller *et al.*, 1990)**. In ζ-deficient cell lines only 5% of normal TCR levels are detected on the cell surface **(Klausner and Samelson, 1991)**. This 5% may be explained by the expression of FcεRI gamma chain homodimers that have been shown to associate with T-cells in place of the zeta chain **(Orloff *et al.*, 1990)**. In summary, like FcγRIII, TCR has a specific signal in its transmembrane domain tagging it for degradation in the ER if left uncoupled to zeta **(Kurosaki *et al.*, 1991)**.

How exactly the FcγR's recruit members of the ζ-chain integral membrane protein family is unclear. Using FcγRI-MANX mutants, lacking

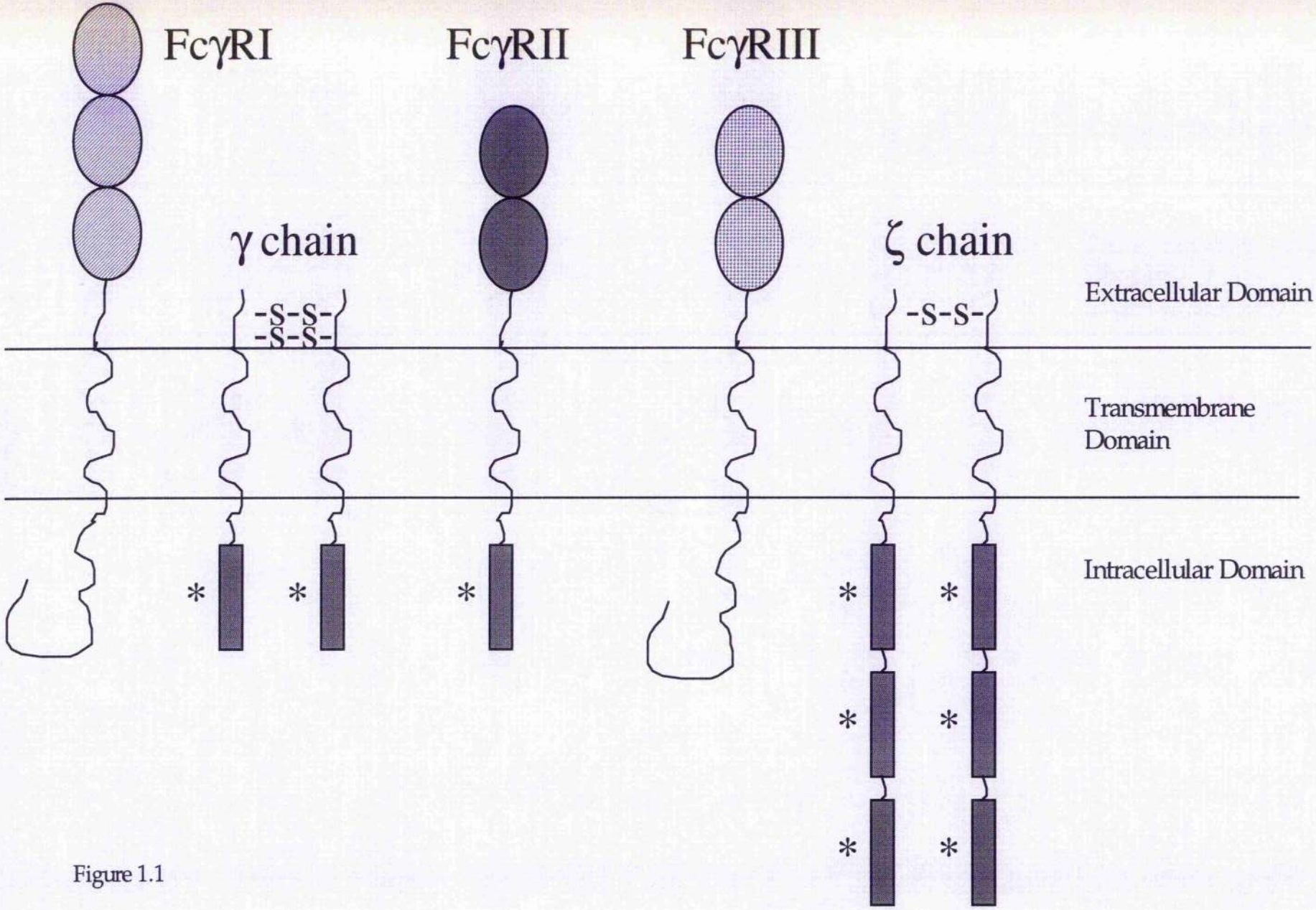

FcγRI
FcγRII
FcγRIII
γ chain
ζ chain
-S-S-
-S-S-
-S-S-
Extracellular Domain
Transmembrane Domain
Intracellular Domain
*

Figure 1.1

Figure 1.1 The three Fcγ receptors, FcγRI high affinity and FcγRII and FcγRIII low affinity. Each receptor is shown next to the signaling subunits to which they most often associate. FcγRII has its own Internal Tyrosine-kinase Activation Motif or ITAM (depicted as an *) and does not require association with either γ-chain or ζ-chain for cell signaling.

the receptor cytoplasmic domain, it was demonstrated that the receptor was still able to recruit cotransfected gamma chain in COS cells. However, the FcγRI-GPI, lacking TM and cytoplasmic domains, was unable to recruit cotransfected γ-chain in COS cells (Figure 1.2). This implicates the TM domain of FcγRI in γ-chain recruitment **(Davis *et al.*, 1995)**. The FcγRI TM domain does have a high homology sequence (MFLVRTVL) with a strategically placed arginine substitution in place of the "crucial" aspartic residue in FcεRI and FcγRIII **(Morton *et al.*, 1995)**.

An interesting comparison can be made between FcγRI and the FcαRI, which binds to ligand surrounded by IgA, the primary antibody in bodily secretions. Like FcγRI, FcαRI associates with the FcεRI γ chain in B lymphocytes. Lacking an endogenous signalling motif this interaction with γ chain is crucial for the FcαR-mediated intracellular signalling **(Morton *et al.*, 1995)**. FcαRI has 5 residues identical and similarly located to the conserved TM residues of the other receptors that lack signalling motifs. Like FcγRI, FcαRI has a positively charged Arg residue in its TM domain that mediates interactions with the γ-chain **(Morton *et al.*, 1995)**. By some unknown mechanism, perhaps the aspartate to arginine switch, FcγRI and FcαR do not require a subunit association for cell surface expression **(Ernst *et al.*, 1993)(Morton *et al.*, 1995)**. This is in contrast to FcεRI and FcγRIII, which require subunit association for cell surface expression.

1.9 Mechanisms of FcR Action: The Internal Tyrosine-kinase Activation Motif

There are important functional similarities in signal transduction between Fc receptor's transducing subunits in diverse cell lineages. The main similarity is the structure of their ITAM, present as an integral part of many distinct receptor subunits, expressed in a variety of cell lines **(Reth *et al.*, 1989)**. The ITAM motif is responsible for the Fc receptor's signalling potential and cellular activation. Although there are variations to the basic motif, ITAM is most commonly found with the amino acid sequence of (D/E) YXXL (X_7) YXXL (D/E). The tyrosines and leucines are required for motif-mediated signalling **(Romeo *et al.*, 1992)(Romeo *et al.*, 1991)**. Interestingly bovine leukaemia virus gp30 and other viruses have been found to express a similar motif to the ITAM, so perhaps it plays a critical role in viral pathology as well **(Cambier, 1995a)**.

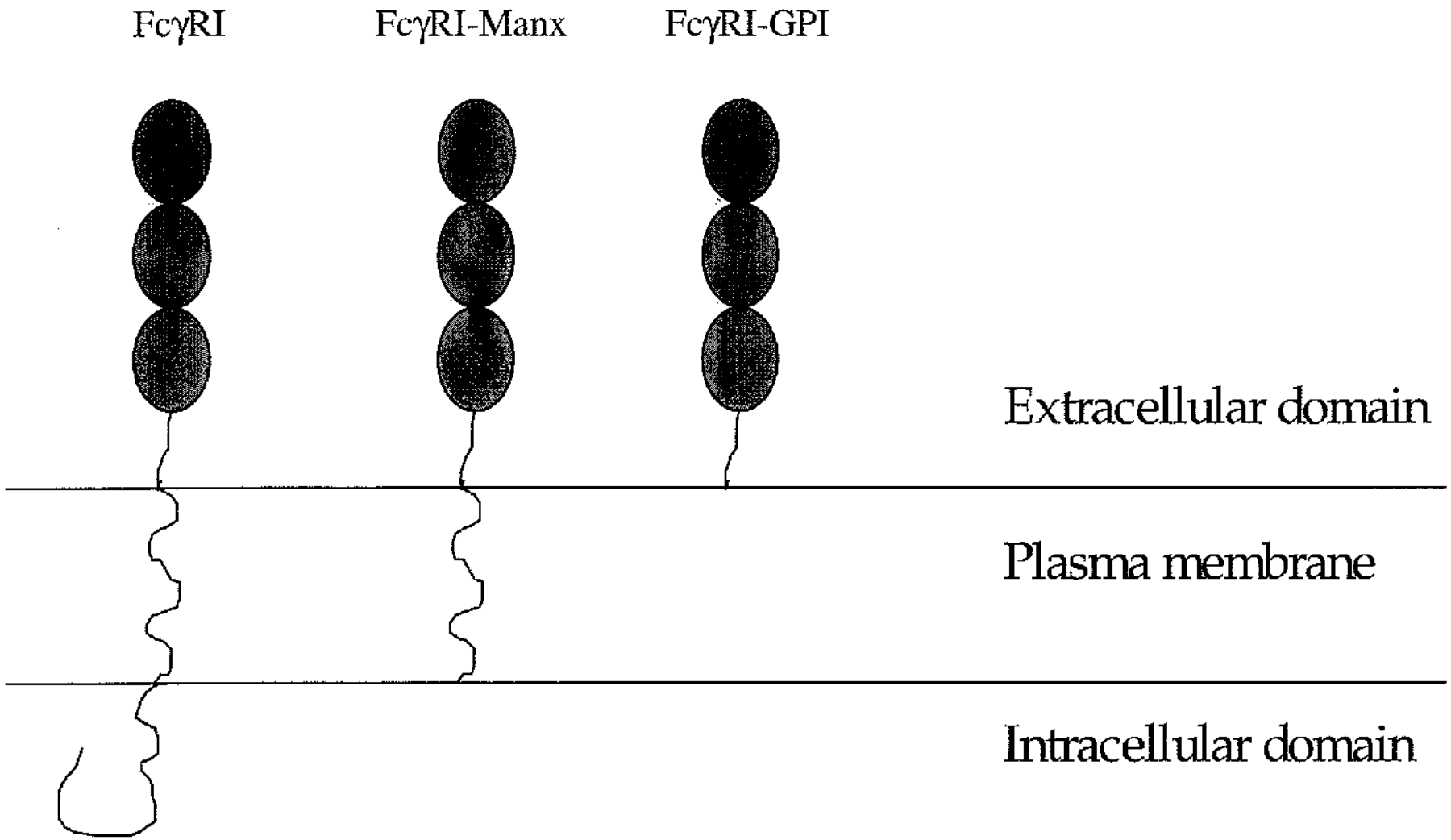

FcγRI
FcγRI-Manx
FcγRI-GPI
Extracellular domain
Plasma membrane
Intracellular domain

Figure 1.2

Figure 1.2 This figure shows the FcγRI receptor alongside mutants FcγRI-Manx (lacking FcγRI's intrinsic cytoplasmic domain) and FcγRI-GPI (lacking both FcγRI's intrinsic transmembrane and cytoplasmic domains). Using these receptors it was shownthat only the extracellular domains of FcγRI were required for FcγRI-mediated endocytosis.

The γ chain has one signalling motif with 7 variable amino acids between the two YXXL boxes (X_7). In contrast, the ζ has three sequential signalling motifs, equally spaced with 10, 11, and 10 variable amino acids respectively between the two YXXL boxes (X_{10}, X_{11}, X_{10}). Anti-CD8 antibody cross-linked chimeric receptors constructed of the CD8 extracellular domains and the ζ chain have shown unequivocally that chimeric-mediated signalling is indistinguishable to that of the full TCR complex **(Irving and Weiss, 1991)(Klausner and Samelson, 1991)**. The zeta chain can be reduced to a minimal 18 amino acid sequence necessary for Ca^{2+} mobilisation (NQLYNELNLGRREYDVL) **(Romeo & Seed, 1992)**. The η splice variant lacks the carboxy tyrosine of the third ITAM, rendering its third ITAM motif non-functional.

FcγRII is unique among FcγR because it has its own intrinsic ITAM and does not need to recruit a ζ-chain family member in order to activate effector molecules. The FcγRII ITAM is similar yet distinct from the ζ/η, and γ chain ITAMs, having 12 amino acids in between the two YXXL boxes compared with the ζ-family 7 amino acids. Again the two tyrosines are the residues of critical importance, and mutation of either destroy signalling potential **(Romeo & Seed, 1992)**.

1.10 Signal Transduction Cascade

Ligand-bound receptors aggregate by an unknown mechanism that allows signalling domains of individual receptor complexes to interact. It was recently found that the FcRn IgG receptor, present in the gut of new born mammals dimerises following IgG binding. In this way IgG from the mother is transported to the bloodstream of the new-born, thus importing passive immunity. The dimerisation was found to involve only ligand bound receptor, implicating FcRn extracellular domains in receptor aggregation following binding. Whether this mechanism will also be observed to be the case for other FcR's has yet to be determined **(Raghavan *et al.*, 1995)**.

Following aggregation ITAM initiation stimulates a cascade of tyrosine kinases that mediate downstream effector function. It has been likened to a scaffold which supports many recruited effector molecules after its phosphorylation. There is also a growing accumulation of evidence that distinct TK phosphorylation patterns are observed in different cells that signal via different ITAM motifs. It is believed that the motifs are likely to have overlapping tyrosine kinase recruitment patterns, and there is an increasing

accumulation of evidence that the signalling pathways which are ITAM initiated are not redundant.

Spacing between YXXL pairs of unique ITAMs facilitate specific TK recruitment patterns **(Weiss, 1993)**. FcγRI, II and III show spacing variation, bringing to light the possibility that different src family kinases initiate signalling cascades after binding distinct ITAM patterns.

1.11 Src Family Tyrosine Kinases

The src family kinases have shown strong associations with ITAM motifs in activated and non-activated receptors. There are 8 members of the src family tyrosine kinases including, Yes, Fgr, Lck, Src, Blk, Fyn, Lyn, and Hck. Different combinations of src proteins have been found to associate with distinct receptor types as both soluble and localised proteins.

Src family proteins all have in common a domain structure including a myristylated N-terminal glycine residue at position 2. This site and a further N-terminal cysteine residue in src-family members leads to palmitoylation and subsequent localisation of src kinases to the inner membrane lipid layer **(Alland *et al.*, 1994)(Koegl *et al.*, 1994)**.

1.12 Importance of SH Domain Binding Sequences

Each src-family protein contains SH1, SH2 and SH3 domains that mediate enzyme activity and enable protein-protein interaction. The amino terminal residues 40-70 are unique between family members, giving the particular kinase differential protein recruiting and signalling capabilities. Distinct regions of the src family may be employed to bind non-phosphorylated versus phosphorylated receptor subunits. SH2 and SH3 domains lack catalytic regions but link phosphorylated receptor ITAMS with cellular effector molecules. Src proteins have an SH4 domain implicated in non-active receptor associations.

1.12.1 SH2 Domains

SH2 domains consist of approximately 100 amino acids and are thought to control intracellular responses by binding to proteins with phosphotyrosines. They are the non-catalytic regions of src-like tyrosine kinases, PLC-γ, and Ras GTPases (guanosine triphosphate activating protein) that bind directly to

tyrosine phosphorylated polypeptides (**Koch *et al.*, 1991**). Post activation src proteins bind preferentially to ITAM tyrosines via their SH2 domains. Tyrosine phosphorylation on Fc receptor subunits creates a pro src-like kinase cleft to which SH2 homology domains of these proteins can bind, forming heteromeric protein complexes near the plasma membrane. SH2 domains are speculated to have a role in modulating protein/protein interactions.

Interestingly, NMR studies have shown that the N and C terminal region of SH2 domains are colocalised in the globular protein structure (**Van der Geer *et al.*, 1994**). The SH2 domain is thus gathered up between the two end residues and left protruding from the structure, free to bind phosphotyrosines. The phosphorylated tyrosines are accommodated by this pocket in the SH2 motif. Phosphorylated SH2 domains initiate full cellular activation.

Importantly, different SH2 domains are better at binding specific motifs on unique signalling proteins (**Songyang *et al.*, 1994**). The SH2 domain of Lck in TCR was found essential to Lck association with ZAP-70 tyrosine kinase and the zeta subunit following activation of the T cell receptor (**Straus *et al.*, 1996**). Lck deficient T cells in knock-out mice fail to show TCR activation events. Also, when critical residues in the SH2 of Lck where mutated to non-functioning residues, the TCR was unable to signal (**Straus & Weiss, 1992**). Seemingly other tyrosine kinases present were unable to rescue Lck-dependent SH2 associations with the TCR.

1.12.2 Importance of SH3 Domain Binding Sequences

The SH3 domain is also involved in control of intracellular responses in activated Fc receptors. It may also have a role in recruiting downstream effector molecules like the p85 subunit of P13K involved in receptor signalling (**Howe & Weiss, 1995**). The SH3 domains are about 60 residues long and like SH2 domains the N and C terminal regions are closely located in the globular structure allowing the binding domain to protrude. Arg and Leu residues in the SH3 domain bind short proline rich peptides in a specific manner (**Van der Greer *et al.*, 1994**).

1.12.3 Importance of SH4 Domain Binding Sequences

An identified N-terminal 30 residues, denoted the SH4 domain, has been mapped by several groups and identified as the location in src-family

allowing it to bind ITAM residues in non-aggregated receptors and receptor subunits **(Cambier, 1995)**. An intermittent SH4 motif -DCSM- is implicated in the binding of src-family tyrosine kinases in non-phosphorylated resting receptors **(Johnson *et al.*, 1995)**.

For example, in the B cell it has been shown that a 10 terminal amino acid sequence of specific src proteins bind to non-phosphorylated receptor **(Pleiman *et al.*, 1994)**. Although the BCR has two subunits, Igα and Igβ, each having a unique ITAM, only the Igα subunit contains the -DCSM- motif that recognizes specific src SH4 domains. Indeed, Igα showed enhanced Fyn and Lyn binding and further Blk binding post-activation, leading to cell stimulation including IL-2 release. In contrast, the Igβ subunit was incapable of stimulating IL-2 production in B cells following receptor activation. When the corresponding tyrosine intermittent motif Igβ -QTAT- was replaced with the Igα ITAMS' -DCSM- motif, IL-2 production was rescued **(Cambier, 1995)**.

1.13 Receptor Associations with Src Family Tyrosine Kinases

Resting receptors associate with src family TKs in a non-active state. However, src family kinases are restricted in resting receptor, even when associated with dimerisation of signalling subunits like ζ–ζ and γ–γ, they are inhibited from transphosphorylation until aggregation of receptors occurs. This simply may involve steric constraints, like distance or orientation, and seemingly the src proteins cannot modify the receptor chain to which they are associated **(Ortega, 1995)**.

Receptor multimerisation results in juxtaposition of cytoplasmic domains and autophosphorylation. Receptor coupling may result in a conformational change leading to mutual transphosphorylation. The change in orientation may result in allowing a better grip of src family tyrosine kinases and permitting cross-phosphorylation of associated src proteins within the same receptor complex and ITAM tyrosines **(Ortega, 1995)**. A similar cross-phosphorylation mechanism is described for the FcεRI receptor **(Pribluda *et al.*, 1994)**.

An alternative to Pribluda's proposed model, Field et al, 1995, demonstrate that p53/56 Lyn associates with resting FcεRI in RBL-2H3 mucosal mast cells and upon activation a rapid increase lyn kinase found associated with this receptor is observed 1 minute after stimulation, and a sharp increase in kinase activity is seen **(Field *et al.*, 1995)**. Whether due to cross-phosphorylation or substantial recruitment of further Lyn protein to the

receptor it waits to be determined how src protein activity is enhanced following activation.

1.14 Syk and ZAP-70 Recruitment

Syk, a 72 kDa protein expressed in B cells, mast cells, basophils and macrophage cells, shares 93% homology with ZAP-70, a 70 kDa protein expressed in NK cells and T cells. Both have been shown to require association with both subunit ITAM sequences and src family tyrosine kinases to potentiate signal transduction **(Chan *et al.*, 1994)**.

Syk family proteins differ in structure from src family proteins in a number of ways. They have no myristoylation signal, therefore they cannot become tethered to the outer membrane like src family TKs. They also don't have a SH3 protein binding domain, and lastly they lack an inhibitory carboxy terminal tyrosine, the point of interaction with regulatory phosphatases on many src family proteins. Also, Syk and ZAP-70 have not just one but two strategically located SH2 domains that both need to be simultaneously phosphorylated for signalling cascades to follow **(Johnson *et al.*, 1995)**.

The main point of contention in unravelling the mechanism by which syk-family kinases become phosphorylated is whether the syk family protein is recruited to the biphosphorylated ITAM, or alternatively is it recruited to membrane bound receptor associated src-family kinases. Previously it was thought that for maximal Syk kinase activation, following receptor aggregation, phosphorylated ITAM tyrosines bound the tandem SH2 domains of a single syk protein **(Pribluda & Metzger, 1992)**. However, in 1993 Weiss proposed two possible mechanisms by which ZAP-70 in particular was recruited and phosphorylated. He speculated that aggregated receptors induce allosteric changes in the cell important for ZAP-70 recruitment via its SH2 domain, where it could then become phosphorylated on its TK-like domain either by src family proteins (Lck/Fyn) or by biphosphorylated ITAM itself **(Weiss, 1993)**. Since then ZAP-70 has been coimmunoprecipitated with Lck, implying a direct association **(Howe & Weiss, 1995)**. Likewise, in activated B-cells Syk binds directly to Blk SH2 domains and complexes with Lyn **(Aoki *et al.*, 1994)**. In FcεRI signalling mechanisms, receptor aggregation causes the activated lyn to phosphorylate the gamma chain which then directly recruits Syk kinase to this domain area **(Shiue *et al.*, 1995)(Kinet *et al.*, 1996)**. There has been some evidence to the contrary, for example, it has

been found that in T cells Lck phosphorylates the dimerised ζ chain subunit in the absence of Syk and all three of the ζ chains ITAMs in turn bind 3 pairs of tandem SH2 domains of ZAP-70 **(Weissenhorn *et al.*, 1996)**. All in all, regardless of whether there is indirect or direct src/syk associations, it is believed optimal signalling of syk family tyrosine kinases is dependent on the type and concentration of src family proteins initiating early signalling events **(Ting *et al.*, 1995)**.

1.15 Summary of Tyrosine Kinase Recruitment

The model so far is presented as follows. Myristoylated membrane bound src-family TKs associate with non-ligated receptor via weak association SH4 domains. When stimulated, aggregation of receptors cause autophosphorylation and transphosphorylation of src-family kinases, which immediately bind and phosphorylate ITAM tyrosines via their SH2 domains. There follows enhanced src-family binding and initiation of syk binding in BCR and FcεRI, ZAP-70 binding in TCR, either by binding uniquely to these ITAMS or through direct interactions with src proteins. Syk family kinases bind via their two SH2 domains, each of which must bind a phosphorylated tyrosine. This association initiates Syk autophosphorylation and distinct signal transduction events inside the cell.

1.16 Phosphatases

Biffen et al. in 1994 found that in the isolated CB1 T-cell line there was a 78% reduction in p56(Lck) associated kinase at the plasma membrane surface. The determining factor in this occurrence was that 40% of the cells did not express CD45 phosphotyrosine phosphatase. In the absence of CD45, CD4&8 associated Lck is hyperphosphorylated on its Y505 residue, abrogating the TCR signalling mechanism, and further Lck recruitment and autophosphorylation is prevented **(Sieh *et al.*, 1993)**.

Normally CD45 acts to dephosphorylate associated Lck protein tyrosine in a regulatory fashion **(Mustelin *et al.*, 1989)**. Indeed, two processes are twined in cell signalling abilities, rapid phosphorylation of receptor ITAMs by non-receptor tyrosine kinases and rapid dephosphorylation by phosphatases. This action strategically couples and then uncouples receptors to downstream signalling molecules **(Paolini *et al.*, 1991)**. Cells lacking CD45 show deviant signalling behaviour in response to stimuli.

As discussed, CD45 has been shown to directly regulate phosphorylation of src family tyrosine kinases **(Mustelin & Burn, 1993)**. Indeed, phosphotyrosine phosphatases have been implicated as TK regulators in many signalling pathways including signalling through the TCR, the insulin receptor, and this area of focus, the FcγR's **(Klausner and Samelson, 1991)**.

In THP-1 cells cocross-linking of mAb to FcγRI &II receptors with anti-CD45 mAb reduced significantly the degree of tyrosine phosphorylation and Ca^{2+} mobilisation in the cell **(Rankin *et al.*, 1993)**. Also, recently in U937 cells the inhibitory mechanism employed by phosphatases on Fc receptor activation was examined using a phosphatase inhibitor, vanadate **(Pfeffercorn *et al.*, 1996)**. It was discovered that when FcγRI γ-chains were cross-linked with specific monoclonal antibodies, if vanadate was added, the kinases activated were not restricted to cross-linked receptors. In this instance kinase activity was detected in the non-aggregated Fcα receptor γ-chains and FcγRII cytoplasmic domains. It seems that phosphatases prevent universal phosphorylation of substrates, only relaxing its regulation of clustering receptor in a vanadate-sensitive manner **(Pfeffercorn *et al.*, 1996)**.

The ability of CD45 to regulate src kinase activity has been explained by Cantley & Koch, 1991. When the carboxy-terminal tyrosine is phosphorylated, the src associates via its own SH2 domain and does not phosphorylate key substrates **(Koch *et al.*, 1991)(Sieh *et al.*, 1993)**. CD45 dephosphorylates this residue increasing its kinase activity substantially, evident by autophosphorylation of tyr-394. In $CD45^-$ cells the Lck tyr-505 is hyperphosphorylated leaving it unable to bind via its SH2 domain other phosphopeptides **(Sieh *et al.*, 1993)**.

1.17 Ca^{2+} Studies

Indication of cellular activation has always been increases in intracellular calcium. Indeed the number of receptors that were cross-linked on the monocyte U937 cell line correlated to the magnitude of the Ca^{2+} response observed **(Davis *et al.*, 1994)**. After receptor engagement, and TK recruitment, the initial biological response is hydrolysis of PI (phosphatidylinositides) following activation of PLC (phospholipase C) **(Klausner and Samelson, 1991)**. In the U937 cell monocyte cell line receptors FcγRI and FcγRII were shown to mobilise PLC-γ upon activation **(Liao *et al.*, 1991)**. Non-receptor tyrosine kinases phosphorylate PLC-γ's two SH2 domain

tyrosine residues rapidly and transiently (**Liscovitch & Cantley, 1994**). Likewise in NK cells PLC-γ1 and γ2 are phosphorylated following FcγRIIIA cross-linking and this phosphorylation is arrested by TK inhibitors (**Azzoni *et al.*, 1992**). This implies that PLC-γ1 and γ2 phosphorylation is accomplished by soluble tyrosine kinases (**Ting *et al.*, 1992**). Finally, in T cells PLC-γ has also been shown crucial to TK-mediated PI turnover happening within 30 seconds of receptor activation (**Klausner and Samelson, 1991**). Again, both Herbimycin A and Genistein blocked PI turnover in T cells, implicating tyrosine kinases in T cell activation (**Mustelin *et al.*, 1990**). In summary FcγRI, FcγRII, FcγRIII, FcεRI, and the TCR receptor are known to couple to non-receptor tyrosine kinases inducing PLC activity. The identity of the specific soluble kinases responsible remains unknown.

Concentrations of Ins-1,4,5-P3 and derived inositol phosphates all increase rapidly following PLC-γ activation (**Liao *et al.*, 1991**). Phosphatidyl 4,5-biphosphate is hydrolysed by PLC-γ into two biochemical messengers, Ins-1,4,5-P3 (inositol 1,4,5-triphosphate) and DAG (diacylglycerol). Ins-1,4,5-P3 elicits Ca^{2+} release from intracellular stores, and DAG activates serine/threonine kinases and PKC (Protein Kinase C).

1.18 Particle Internalisation: Endocytosis and Phagocytosis

Endocytosis involves internalisation and destruction of non-opsonised particles. Phagocytosis is the process by which opsonised immune complexes are internalised and destroyed.

1.19 Endocytosis

Endocytosis is the process by which receptor bound ligand is internalised and either recycled back to the cell surface or retained within endosomal compartments of the endocytic cell. The mechanism of endocytosis of FcγR's has been shown to vary between the different Fc receptors, FcγRI endocytosis being a TK independent process while FcγRII endocytosis being a TK dependent process.

1.19.1 FcγRI and FcγRII-Mediated Endocytosis

When FcγRI binds monomeric IgG, the receptor disengages from the cytoskeleton by disengaging from ABP-280 (actin binding protein, non-

muscle filamin). The identity of accomplice proteins linking FcγR's with ABP's are unclear. There are several candidates that will be discussed in the phagocytosis section. However, when this connection is achieved it allows the ligand/receptor complex to be internalised.

Receptor cross-linking is not essential for ligand internalisation. It was previously thought that specific receptor proteins are targeted to active cytoplasmic tail tyrosine residues of ligand-bound receptors such as FcγR's enabling endocytosis **(Pearse & Robinson, 1990)**. However, seemingly when the FcγRI/ABP complex is disrupted, the IgG/FcγRI complex interacts with accessory molecules in an ITAM independent manner. This specific recognition allows structural changes of the membrane, resulting in efficient particle engulfment.

Only the extracellular domains of the receptor are involved in FcγRI-mediated endocytosis **(Harrison *et al.*, 1994)**. A FcγRI-GPI mutant lacking both TM and cytoplasmic domains of FcγRI, was able to maintain endocytic ability in COS cells **(Davis *et al.*, 1995)**. In this way the untethered receptor complex is internalised and then rapidly recycled from endosomal compartments **(Bonnerot & Amigorena, 1993)**.

In COS cells genistein, a TK inhibitor, is unable to hamper the FcγRI endocytic process **(Davis *et al.*, 1995)**. Using primaquine, which blocks plasma membrane passage of immune complexes, non-cross linked cells were found to have accumulated immune complexes inside primary endosomal compartments. This demonstrates the recycling ability of non-cross-linked FcγRI. Removal of primaquine resulted in non-degraded antigen being recycled to the cell surface **(Harrison *et al.*, 1994)**. Therefore, FcγRI mediated recycling is ligand dependent and tyrosine kinase independent.

After ligand binding if receptor cross-linking occurs the recycling pathway is diverted and the already untethered receptor complex is directed in a TK dependent process into deep lysosomal compartments where it is degraded **(Bonnerot & Amigorena, 1993)(see phago section)**.

In contrast, endocytosis and receptor recycling mediated by FcγRII and FcγRIII on phagocytic cells requires TK activation, and is inhibited fully by genistein **(Ghazizadeh *et al.*, 1994a)(Harrison *et al.*, 1994)(Amigorena *et al.*, 1992)**. While the extracellular domains of FcγRI alone are capable of initiating a recycling pathway, the extracellular domains of FcγRII seem unable to mediate the same process. A mutant substituting FcγRII extracellular domains with those of FcγRI restored TK independent endocytic ability,

demonstrating the importance of the extracellular FcγRI domains (**Davis *et al.*, 1995**).

1.20 Phagocytosis

While endocytosis and phagocytosis both involve antigen internalisation, phagocytosis is restricted to internalisation of particles > than 1 mm and the process is inhibited below 18^0C. It also necessitates paxillin induced actin polymerisation and pseudopodia formation.

Phagocytosis is critical to an organism's defence against infectious agents. It is a complex process involving specialised recognition receptors like FcγR's, on the surface of the phagocytic cell. It also involves dynamic cytoskeletal changes and exchange of phagosomal components with intracellular compartments. In areas of infection professional phagocytes, PMN, accumulate and are differentiated generally into macrophages. The macrophage lumen houses toxic components which are derived from the superoxide ion (0^{-2}). The end components of an oxidative burst include hydrogen peroxide (H_2O_2), the hydroxy radical (HO^-), and hypochlorous acid (HOCl) (**Bokoch, 1995**). The oxidative burst facility and ability to phagocytose enables the macrophages to effectively control the multiplication of infectious agents.

1.20.1 Subunit Recruitment

For phagocytosis, FcγRI and FcγRIII need to recruit γ-chain, ζ-chain, or the cytoplasmic portion of FcγRII (via its TM portion), that supplies it with the machinery necessary for internal signalling (**Amigorena *et al.*, 1992**)(**Davis *et al.*, 1995**)(**Indik *et al.*, 1994a**). FcγRI without γ-chain is only able to recycle via a TK independent pathway.

Studies where FcγRI-γ extracellular domains are replaced by CD2 which binds LFA-3 on SRBCs are still capable of phagocytosis. Likewise, when the extracellular regions of FcγRII are replaced with CD2, the indigenous TM and IC domains alone were sufficient for phagocytosis. Receptor aggregation causes ITAM clustering connecting signalling motifs in the cytoplasmic domain. This activation process in both FcγRI-II and FcγRI-γ receptors is sensitive to genistein. Therefore, both FcγRI-γ and -II mediated phagocytic effector function is ligand independent and TK dependent (**Harrison *et al.*, 1994**)(**Davis *et al.*, 1995**).

1.21 Models of Phagocytosis

As described, the endocytic recycling pathway is diverted to a retention pathway following FcγRI cross-linking **(Harrison *et al.*, 1994)**. The initial phagocytic event is pseudopodia formation, when tentacles trap cross-linked antigen. The complex is then transported to phagosomes and degraded.

As early as 1975 contractile proteins in the cytoplasmic domains of non-muscle phagocytic cells had been discovered, but even today the exact contractile elements necessary for particle engulfment remains unclear **(Griffin et al., 1975)**. Actin, actin binding protein, and myosin are all elements redistributed following cellular activation and pseudopodia advancement in phagocytic cells **(Valerius *et al.*,1981)**. There were two proposed models by which pseudopodia extension proceeds, the trigger model and the zipper model **(Swanson & Baer, 1995)**(Figure 1.3).

1.21.1 Zipper Model

While exploring the nature of particle engulfment Griffin et al in 1975 found that when phagocytosis of a particle was initiated, close by or "observer particles" attached to the macrophage surface were not ingested coincidentally. This suggests that particles capable of initiating a phagocytic signal only do so in membrane domains in direct contact with that particle. Likewise intracellularly accumulated ligand are contained within vacuoles that reflect their diameter sizes **(Griffin *et al.*, 1975)**.

Griffin et al in 1995 tested the ability of peritoneal macrophages to ingest partially opsonised particles. They accomplished this by two distinct methods. Firstly they trypsinised already bound particle so antibody was stripped from all regions albeit the interface between plasma membrane and the particle. Secondly, they used anti-macrophage IgG to block Fc receptors that were not situated at the plasma membrane and particle interface. At physiological temperature in both experiments phagocytosis was blocked. Although it was suggested that this may be explained by the fact that insufficient receptor antigen connection debilitated a triggered threshold level of interaction from being reached, Griffin concludes that these experiments lend support to the Zipper mechanism of phagocytosis.

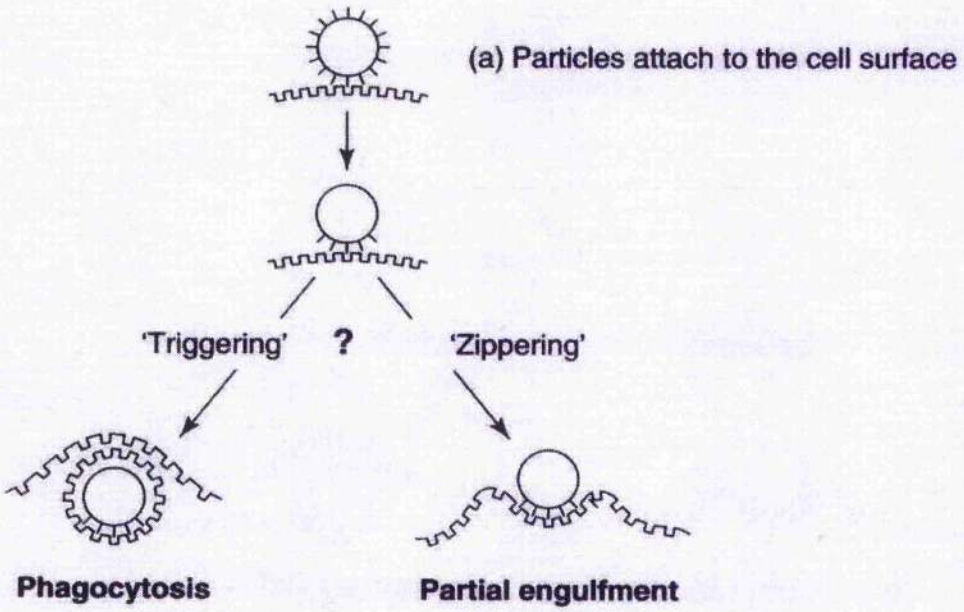


Figure 1.3 The alternative zipper and trigger models of phagocytosis, taken from Swanson and Baer, 1995. In the zipper model internalisation requires direct contact between opsonins (indicated by spikes) and receptors (indicated by square pits). In contrast, in the trigger model initial binding allows for internalisation of particle in an "all or nothing" event.

All of these observations support the idea of a phagocytic segmental response that is proportional to the size of the particle attached to it. This suggests a "zipper" motion of cell pseudopodia surrounding the receptor attached particle, and complete ingestion will only occur if the particle is 100% opsonised so receptor can bind the whole circumference of the particle.

1.21.2 Trigger Model

While the zipper model proposed by Griffin and Silverstein is one involving local control, where continual attachment of new receptor is required for pseudopodia advancement the triggered response postulates a mechanism by which a specific stimulus of set magnitude stimulates a phagocytic response. In this model it is postulated that the concentration of opsonised antigen in contact with the phagocyte's membrane will reach a threshold amount after which phagocytosis will proceed **(Swanson & Baer, 1995)**. This all or nothing "triggered" phagocytic response would parallel an action potential in neuronal cells. Support for this mechanism is seen in epithelial cells reacting to the presence of the S.*typhimurium*. bacteria. In response to colony stimulating factor, membrane ruffling occurs that can be described as advancement of pseudopodia. Indeed, stimulation of the epithelial cells with S.*typhimurium* bacteria has been shown to induce such "unguided" pseudopodia extension, resulting in bacterial uptake. The effect is not localised like in the zipper model, as shown by passive uptake of particulate matter in the vicinity of the S. *typhimurium*. including other bacterial species and latex particles **(Swanson & Baer, 1995)**.

1.22 Linking Signalling Molecules to Actin Rearrangements

The process of phagocytosis of FcγRI/γ-chain receptors and FcγRIIa receptors are both not ligand-specific processes **(Hutchinson *et al.*, 1995)**. Only receptor aggregation is necessary for ITAM phosphorylation and the resulting TK recruitment. This was shown when extracellular domains were replaced with CD2 adhesion molecule that can bind LFA-3 of sheep red blood cells. When the receptor cross-linked, the transmembrane and intracellular domains were equally as capable of activating effector molecules in COS-7 cells **(Hutchinson *et al.*, 1995)**.

Post aggregation signalling molecules have the important role of targeting intracellularly accumulated ligand to lysosomal compartments for

degradation. The associated proteins responsible for linking Fc activation with underlying changes in the cytoskeleton awaits to be elucidated. The importance of TK activation is becoming more and more clear.

1.23 Importance of Syk in Mediating Phagocytosis Events

All phagocytes demonstrate tyrosine phosphorylation on receptors in the phagocytic cup forming region **(Greenberg *et al.*, 1994)**. The TK inhibitor Genistein prevents phagocytosis. However, it has been demonstrated that Fc mediated tyrosine kinase phosphorylation patterns were not altered in mouse macrophages when actin polymerisation was blocked using cytochalasin D **(Greenberg *et al.*, 1994)**. This leads to speculation that TK activation proceeds the recruitment of actin polymerising regulator proteins.

1.24 Other Important Signalling Proteins Involved in Phagocytosis

There are other interesting proteins besides Syk that may signal to actin regulatory proteins. Briefly other components that may play a role in actin rearrangements include, PKC and phospholipid modifying enzymes like P13-kinase and GTP-binding proteins.

The PKC family of protein kinases is activated following Fc antigen stimulation of Fc receptors, although its role remains to be tested. When complement coated particles "sink" into the membrane via non-Fc receptors, there is some evidence that it is not a tyrosine kinase dependent process, yet requires PKC activity **(Allen *et al.*, 1996)**.

P13-K when activated phosphorylates phosphatidyl inositol-4-P and phosphatidyl 4,5-P2 and may be directly involved in cytoskeletal rearrangements or may activate small GTP-binding proteins that are involved **(Greenberg *et al*, 1995)**. It was recently discovered that PI3-kinase p85 regulatory domain binds to the SH2 and SH3 domains of the src-family proteins Ick and Fyn **(Susa *et al.*, 1996)**. It is possible that mechanisms like membrane ruffling via Fc receptors in macrophage cells is regulated by the P13K tyrosine kinase. PDGF induced membrane ruffling has been shown to require P13K activation **(Van der Greer *et al.*, 1994)**.

Lastly GTP-binding proteins may be involved, for example Rac 1 microinjection induced membrane ruffling in phagocytic cells. As mentioned

Rac1 interacts directly with P13-kinases non-catalytic domain 85kDa, suggesting its important influence on phagocytosis as a downstream element of P13-kinase. Interestingly Ras also plays a role, having been found to interact with P13K's 110 kDa catalytic domain **(Greenberg *et al*, 1995).**

1.25 Immediate Actin Regulator Proteins

A massive zone of polymerised filaments is found localised in the cytoplasm underneath the ingested particle. Clusters of different polymerising regulator proteins are found evenly distributed in the area those being, paxillin, annexins, and ponticilin **(Allen *et al.*, 1996).** However, the protein that provides the pivotal link between Fc receptors and pseudopodia formation following their activation remains unclear.

After the signalling domains of Fc receptors aggregate and phosphorylate Syk 72, phosphorylation of the cytoskeletal-associated protein paxillin (68 kDa) closely follows. This ITAM phosphorylated paxillin accumulates under macrophage pseudopodia. Annexin I has also been shown to have an increased degree of phosphorylation following receptor associated ITAM activation **(Van der Greer *et al*,. 1994).** Another possible candidate, ponticilin, has been shown to be responsible in Dictyostelium for F-actin binding, and talin, found in accumulation under FcγR-stimulated nascent phagosomes **(Greenberg *et al*, 1995).** Interestingly, interactions between the cell surface and the underlying actin based cytoskeleton have been shown to be ponticilin-mediated although further analysis is critical **(Hitt *et al.*, 1994).**

1.26 Aims of Experimentation

The aim of this study was to elucidate differential patterns of tyrosine kinase phosphorylation for distinct FcγR signalling domains using gamma (IFNγ) and dibutyl cAMP (DBC) treated U937 monocyte-like cells, upregulating FcγRI + γ chain of the FcεRI and FcγRII receptors respectively.

To further examine differential recruitment by distinctive ITAMs, a chimeric receptor was made by fusion of the extracellular region of FcγRI to the transmembrane and cytoplasmic tail of zeta chain. This chimeric receptor was then compared to other similarly constructed mutants, FcγRI-γ and FcγRI-II for its ability to phagocytose sheep red blood cells (SRBC) in the presence and absence of specific tyrosine kinases.

Chapter 2

Materials and Methods

2.1 General Supplies

Sigma Chemical Co., Fisons (now Fischer) Scientific Equipment and BDH Merck supplied the lab with both general stock solutions and equipment. Boehringer Mannheim was a main supplier of biochemically active enzymes, and suppliers of more specific equipment and services are detailed in the appropriate section. The components of solutions used in the materials and methods of both Chapters 2 &3 are listed in full in Appendix 1&2 respectively. All solutions and heat and pressure resistant lab equipment were sterilised by autoclaving, heating to a temperature of 121^0C with a pressure of 1.05 bar for 20 minutes.

2.2 Mammalian Tissue Culture

Laminar flow tissue culture hoods used for mammalian cell culture were supplied by Microflow Laminar Flow Cabinets (MDH Ltd.). All tissue culture cells were maintained at 37^0C with 6.5% CO_2 and 100% humidity in a Napco® Model 5410 incubator. All solutions used in tissue preparations were sterilised by autoclaving or filtration through 0.22µm filter units by Millex-GV, Millipore.

2.2.1 COS-7 Origin

The COS 7 cell line was derived from the kidney cells of the African Green Monkey **(Gluzman, 1981)**. To maintain system homogeneity, U937 cells are the choice lineage used to examine signalling events associated with Fc receptor stimulation, however, COS 7 cells are an invaluable tool for the examination of components involved in Fc-mediated endocytosis and phagocytosis **(Harrison *et al* 1994b; Davis *et al* 1995)**. COS 7 cells, being of a non-haemopoietic lineage, do not express indigenous Fc receptor groups. However, the cell line is capable of high surface expression of transiently expressed receptor cDNAs (and tyrosine kinase cDNAs) so the phagocytic

and endocytic events mediated by individual receptors can be observed in isolation from the effect of other isoforms of the receptor family.

2.2.2 Cell Culture of COS-7 Cells

COS-7 cells (obtained from Brian Seed) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplied by Gibco BRL. The medium was supplemented with 10% calf serum (Gibco BRL), glutamine (2mM), gentamicin (15 μM), penicillin (100 IU/ml) and streptomycin (100μg/ml).

To maintain COS-7 cell cultures, cells were split every 4-5 days into sets of new disposable polystyrene (Costar®) plates. This was done by washing the cells with 2 x 5ml of PBS (Appendix 1) while they maintain cohesive attachments to the original plate. Then 1ml of trypsin (Appendix 1) is added to the plate and rotated until the solution covers all areas of cells. The excess trypsin is aspirated off and the typsinized plate is incubated at 37^0C for 10 minutes in which time the residual trypsin lifts COS-7 cells from the plate surface. The cells are then suspended in 10% calf medium and divided onto fresh plates, typically there is a 1:6 division of plates and typically 10ml of medium per plate.

2.2.3 U937 Origin

To investigate signalling through Fc receptors types I and II the histiocytic lymphoma cell line U937 was used. In this monocyte-like cell line both FcγRI and FcγRII are present while FcγRIII is absent on the cell surface. When treated for 48 hours with dibutyl cAmp the cell line differentiates into a more macrophage like state, inducing up-regulation of the FcγRII receptor type and down-regulating both FcγRI and γ-chain surface expression **(Sheth *et al.*, 1988)**. In contrast, in response to 24 hour treatment by a multitude of cytokines (here IFN-γ was used exclusively) U937 cells are triggered to differentiate into a state of activation which includes up-regulation of FcγRI and γ-chain, down-regulation of FcγRII expression, and subsequent increase in active cell-state responses, like phagocytosis **(Guyre *et al.*, 1983)(Ernst *et al.*, 1993)**.

2.2.4 Cell Culture of U937 Cells

U937 cells were cultured in RPMI medium supplied by Gibco BRL. RPMI medium was supplemented with 10% Fetal Calf Serum, 2mM glutamine, 10mg/ml penicillin, and 10mg/ml streptomycin.

Every 4-5 days the U937 cells were harvested into a Sarstedt tube and centrifuged for 3 minutes at 3,000 rpm in a Sorvall RT6000B benchtop centrifuge. The supernatant was removed and the cells were resuspended in fresh medium and divided in even aliquots between 5-6 flat bottom flasks (Costar®). The flat bottom flasks were left with loose tops at 37^0C.

2.2.5 U937 Cell Quantification

Cell counts of viable U937 cells were conducted using a haemocytometer (Sigma). The sample of 50ul of cells were diluted 1:1 with phosphate-buffered saline solution (PBS)/Bromophenol Blue (pinch), to stain the cells. In this way cellular debris and dead cells were stained blue, as the bromophenol blue readily passed through their punctate membranes, while the more resistant live cells remained translucent. The suspension was transferred by pipette to the haemocytometer by carefully letting a drop of material touch the edge of the cover slip covering the grided section of the apparatus. Through capillary action the droplet was redistributed in a relatively uniform way over the counting grid. Translucent cells were counted in the middle and four corner squares. The average number of cells per millilitre was calculated by taking the average number of cell counts per square, accounting for the dilution factors involved, and then multiplying by 10^4.

2.2.6 Total Protein Quantification in U937 Cells

A Bradford assay was used to quantify protein in these experiments. A calibration curve containing 5-40 μg BSA was set up using Bovine Serum Albumin (Sigma) as the standard protein. To each 300μl of supplied stock 5μl of sample was added. After 30 minutes at 37^0C the samples were assessed at 595nm (DU-67, Beckman) and absorbance values were assessed by comparisons of standard BSA concentrations graphed as a standard curve.

2.3 Photography

A Nikon PFX Labophot-2 microscope was used to obtain photomicrographs at 40 times magnification. Photographs were developed at the University of Glasgow photography department.

2.4 Bacterial Culture

All bacterial culture work was performed on the bench top cleaned with 70% ethanol. Solutions, media, and heat resistant equipment, i.e., glassware and plasticware were all autoclaved before use. A bunsen burner was used to sterilise the ethanol dipped glass spreading rod prior to immediate use. To maintain aseptic conditions sterile solutions and media were flame sterilised, following the protocol in Maniatis, before and after exposure to the open air. Bacteria cultures spread onto agar plates were grown in inverted position at 37^0C in an incubator (Napco Model 301). Two bacterial strains were principally used, those being TG1 and MC1061/P3 *E. coli* strains. TG1 was used in combination with an ampicillin resistant eukaryotic expression vector pSVL (Pharmacia. GenBank Accession Number:- U13868) and the pTag vector (Invitrogen). The MC1061/P3 was used in combination with the expression vector CDM. This vector system is both ampicillin and tetracycline resistance **(Seed and Aruffo 1987a)**. Amber mutations in the P3 vector disrupt its antibiotic resistance ability. Bacteria transformed with the CDM vector possess a mutant tRNA that recognises the susceptible P3 codons and inserts amino acids that confer antibiotic resistance.

TG1 F'*traD36 lacI*^{q} *Δ(lacZ)M15 proA*$^+$*B*$^+$*/supE Δ(hsdM-mcrB)5* ($r_K^-m_K^-McrB^-$) *thi Δ(lac-proAB)*
(Sambrook *et al* 1989)

MC1061/P3 F$^-$ *araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL* (Str^r) *hsdR2* ($r_K^-m_K^+$) *mcrA mcrB1* [P3*kan*^{r} amber *amp*^{r} amber *tet*^{r}]
(Sambrook *et al* 1989)

2.4.1 Media Preparation

All plasmid work done involved transfection into *E. Coli* bacteria. TG1 and MC1061 p3 strains were grown in Luria-Bertani media (LB, Gibco BRL). In one litre of distilled water, 20g of premix LB concentrate was added and mixed. The mix was then autoclaved. When the medium had cooled to a

temperature under 50^{0}C antibiotic was added. In the case of medium being used to grow TG1 *E. coli*, 100μg/ml of ampicillin (Sigma) was added. In the case MC1061/P3 *E. coli*, transformed here exclusively by the CDM vectors, this two vector system has weaker antibiotic resistance, therefore 12.5μg/ml of ampicillin and 7.5μg/ml of tetracycline (Sigma) were added to the cooled medium.

Agar plates were prepared using 32g of premixed LB agar powder (Gibco BRL) in 1 litre of distilled water. The mixture was autoclaved and after cooling, the same relative concentrations of antibiotic were added as stated above. The agar plates were then allowed to congeal at room temperature with covers in place and then inverted in the 37^{0}C incubator for 30 minutes or more with covers removed, so that the condensation can evaporate from the agar surface and plate walls. Plates were either used immediately or stored for 1-2 weeks at 4^{0}C.

In the case of bacterial colonies selected by a vector containing the beta-galactosidase gene in the poly-linker region, sterile LB agar 100mm plates were coated with 2.4mg of the inducer isopropyl-β-D-thiogalactopyranoside (IPTG) and 1mg of the chromogenic substrate 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal). The plates were left for 30 minutes at 37^{0}C to absorb the components before use.

2.4.2 Competent Bacteria

The calcium chloride method was used to render all bacterial strains competent to absorb DNA. Both TG1 and MC1061/P3 strains were individually streaked onto plates and grown overnight at 37^{0}C, although the plates MC1061's were streaked on contained 50μg/ml kanamycin to maintain the integral P3 plasmid. A single colony from each was then transferred into 5 ml of TYM medium and was shaken by rotation at 37^{0}C for 3 hours. The culture was then added to 500ml of TYM and allowed to grow while shaking at 37^{0}C. When the absorbency of the suspension was 0.5 OD units at 600nm the solution was snap cooled in ice water and centrifuged in sterile polypropylene tubes at 5,000rpm for 10 minutes at 4^{0}C (GS-3 rotor, Sorvall centrifuge). To wash the pellet about 50 ml of ice cold TfbI was used to resuspend the pellet. After a 5 minute incubation on ice the mixture was again centrifuged at 5,000rpm for 10 minutes at 4^{0}C. Now the pellet was resuspended in 20ml of ice cold TfbII and aliquoted into 200μl and 500μl

amounts per eppendorf. The samples were incubated on ice for 15 minutes before being snap-frozen in liquid nitrogen and stored at -70^{0}C.

2.4.3 Transformation Technique

Competent cells were thawed from the -70^{0}C on ice and aliquoted into 50µl samples, one for each plasmid DNA prep waiting to be transformed and one control aliquot. Plasmid DNA was added to the aliquot of competent cells at a concentration of 10 to 100 ng of DNA (usually 1-10µl) and mixed gently. After a 30 minute incubation on ice all samples, including control, were heat shocked at 42^{0}C for 2 minutes. The samples were then placed back on ice for up to 2 minutes followed by the addition of 1 ml of LB media (no antibiotics) to each eppendorf. A 1-2 hour incubation period followed after which 100-200µl of each sample were plated, using a flamed glass rod, onto 100mm bacterial plastic Petri dishes agar plates, prepared with selection specific antibiotics and compounds. The control bacterial samples were plated, allowing for determination of antibiotic effectiveness. The plates were left overnight at 37^{0}C in inverted position and checked in the morning for colony growth. Often plates were left for over 16 hours for MC1061 transformed bacteria because they are slow growers.

pSVL and pTag transformed TG1 bacteria were grown on ampicillin plates only while CDM transformed MC1061/P3's have conferring bacterial resistance to both ampicillin and tetracycline. Successful recombinants containing vectors that were designed to interrupt the β-galactosidase gene grew as white colonies on LB-ampicillin/IPTG/X-Gal plates. On negative control plates β-galactosidase reacted with X-Gal to produce blue colonies of unsuccessful recombinants. Successful colonies were screened by conducting minipreparation on positive growers, and doing restriction digests on the bulk plasmid DNA collected after this process. Digests were electrophoresed and plasmid band sizes present were used as determinants of successful recombination products.

2.5 Nucleic Acid Isolation Techniques

2.5.1 RNA

"Finger Nuclease" contamination was prevented by wearing gloves at all instances where RNA work was taking place. On a workbench designed for

the manipulation and isolation of RNA, general RNA handling procedures were used. All disposable plastic wear, such as pipette tips and eppendorf tubes were autoclaved and used exclusively for RNA work. To inactivate nucleases, glassware and metal equipment used was baked at 270^{0}C for eight hours. The ribonuclease inhibitor, diethylpyrocarboate (DEPC) was used at a 0.05% concentration to treat all solutions used in RNA involving reactions. DEPC was left in relevant solutions overnight, which were then autoclaved in the morning to deactivate the remaining DEPC. DEPC treated water was used wherever possible, i.e. reaction buffers and stock chemical dilutions, and was also used to clean RNA- exclusive electrophoresis apparatuses.

2.5.2 RNA Extraction

Using guanidinium isothiocyanate (GuSCN)/acid phenol, cellular RNA was isolated from whole cells homogenates. A Jurkat p10 T-cell suspension was centrifuged at 4^{0}C for 3 minutes at 900g. The supernatant was removed and the cell contents were resuspended in 5 ml of PBS. This washing step was repeated two times. The cells were once again pelleted and guanidinium isothiocyanate was added. The sample was homogenised through vortexing and 0.1 volumes of 2M sodium acetate, pH4 was added to acidify the homogenate. The acidified layer was then extracted to remove protein from the homogenate. This was done through the addition of 1 volume water-saturated phenol (Rathburn Chemicals Ltd) and 0.2 volumes of chloroform: isoamyl alcohol. The RNA contained in the aqueous phase was removed. To precipitate the sample 1ml of 100% ethanol was added to the sample which was then frozen overnight at -20^{0}C.

The next day the samples were spun at 4^{0}C for 30 minutes at 8000g. The sample was resuspended in guanidium isothiocynate and the process above was repeated. After the overnight reprecipitation with 100% ethanol the sample was spun down again as described above and resuspended in ribonuclease free water. The samples were stored at -70^{0}C.

2.6 Plasmid DNA

2.6.1 Plasmid Minipreparation (modified alkaline lysis method)

Individual vector containing colonies were selected from agar plates using a yellow pipette tip, and were placed into 30ml sterile glass test-tubes 2ml of LB

with the appropriate concentration of vector specific antibiotics. The inoculations were left to shake vigorously overnight at 37^{0}C. One ml of each culture was then aliquoted into 1.5ml eppendorfs microfuge tubes and spun down for 1 minute at 12,000rpm at a temperature of 4^{0}C. The supernatant was aspirated and the resulting pellet was put immediately on ice. The pellet as resuspended in 100µl of ice-cold GTE (made using 10mM EDTA) with 4mg/ml chicken egg white lysozyme, and stored at room temperature for 5 minutes. The cells were then lysed using 200µl per sample of 0.2 M NaOH/ 1% SDS. After inversion mixing and 5 minutes incubation on ice, proteins were precipitated from the lysed cells using 150µl of ice-cold 5 M potassium acetate/5 M acetic acid solution (pH 4.8). The samples were mixed by inversion and left on ice for 5 minutes before being spun at 12,000rpm for 5 minutes. The supernatant was transferred to a fresh tube containing 500µl of phenol/chloroform (pH 8.0; buffered with 0.1M Tris-Cl) per tube. The mixture was vortexed thoroughly and spun for 5 minutes at 12,000rpm. The top aqueous layer was removed and placed in another fresh eppendorf containing 900µl of 100% ethanol. After vortexing, the mixture was left to stand at room temperature for 2 minutes and then spun at 12,000rpm for 10 minutes. The 100% ethanol was removed and the pellet was washed in 70% ethanol and left overnight at -20^{0}C for maximum yields. The next day it was spun for 15 minutes at 12,000rpm after which the supernatant was removed and the pellet was air-dried or sped dried in a 37^{0}C Dri-Block (Techne) left with cap open for 15-20 minutes. The pellet was resuspended in 20µl of TE buffer containing 10µg/ml of RNase A which had been pre-boiled to deactivate DNase. The DNA concentration was quantified and then the plasmid DNA was stored for further use at -20^{0}C.

Minipreped samples now contained µg amounts of DNA that could be tested to see if they contained the correct manipulated sequence of mutant plasmids. This was done by doing diagnostic restriction digests on 2µl of the DNA, and if bands of the desired size were produced on 5cm x 7cm agarose gel, the positive plasmids were then amplified by the maxi-preparation procedure.

2.6.2 Plasmid Maxipreparation

Maxipreped samples can have up to 1mg of plasmid DNA present of much higher purity than solely minipreped samples. These pure, large scale

amounts of plasmid DNA are ideal for subsequent sequencing and mammalian cell (COS-7) transfection experiments.

For High Copy Plamids and Low Copy Plasmids the following protocol for achieving large scale preparation was used. An individual positive colony (in the case of TG1s), or a streak of several colonies (in the case of MC1061/P3), was grown in 30 ml sterile glass tubes with 2 ml of LB broth, containing the standard amount of the appropriate antibiotics. The seed cultures were grown for 6 hours while shaking and were then used to inoculate an overnight 500ml culture, again containing the standard amount of the appropriate antibiotics, which was left on the orbital shaker at 37^0C overnight. The cells were then pelleted by centrifugation at 5,000rpm (Sorvall centrifuge with a GS-3 rotor) for 10 minutes at room temperature in 500ml centrifuge tubes. The supernatant was removed and 18mls of cold 10mM EDTA solution was used to resuspend the pellet by pipetting up and down. 36 ml of fresh 0.2 M NaOH/ 1% SDS lysed the bacteria cells with gently mixing, and 18 ml of 5M potassium acetate/5M acetic acid (pH 4.8) was added followed by short-term vigorous shaking to precipitate proteins out from the aqueous layer. After a 5 minute incubation on ice, the samples were spun for 10 minutes at 5,000rpm and 4^0C. The plasmid DNA containing supernatant was transferred to a fresh 500ml centrifuge bottle through a doubly layered muslin/cheese-cloth filter, removing any remnant protein pellets. An equal volume of isopropanol was added and mixed vigorously to precipitate the DNA and remove RNA which does not precipitate under these conditions. The bottles were respun at 5,000rpm and 4^0C for 10 minutes. The supernatant was removed and the pellet was washed with 10 ml of 70% ethanol, making the white pellet visible on the bottom of the centrifuge tube. Excess ethanol was removed and the pellet was air-dried and then resuspended in 3ml of TE buffer.

2.6.3 Caesium Chloride Purification Method

Plasmid DNA was isolated and purified using the caesium chloride method. Four grams of Caesium Chloride was weighed out and added to the resuspended pellet. Addition TE buffer was added to the DNA pellet until the final volume was 3.5 ml to which 250µl of Ethidium Bromide at 10mg/ml was added. The TE resuspension must be kept at room temperature otherwise the CsCl will crystallise, and may rupture the Beckman centrifuge tubes. The mixture was pipetted into 3.9ml Quick-Seal™ polyallomer tubes

(Beckman) and heat sealed. The density of the mixture in the sealed tubes was 1.55g/ml, so the tubes weighed 7g ± 0.1g. The tubes were placed in a TLN 100 rotor (Beckman) in a balanced position with spacers and then caps used to secure their positions. The Beckman Optima^{TM} ultra-centrifuge was spun at over 18^{0}C overnight at 55,000rpm. After 16-18 hours a visible closed and circular DNA plasmid band could usually be seen below protein and nicked, linear plasmid DNA bands, and above an RNA pellet. A blue type 21G Microlance-3^{TM} (Beckton Dickinson) needle was used to puncture the tube top allowing for ventilation, while a green type 21G Microlance-3^{TM} (Beckton Dickinson) was used to extracted 1-2ml of the closed circular plasmid DNA band into a 5ml disposable syringe (Beckton Dickinson). The needle was removed from the syringe (to prevent shearing of DNA) and the syringe contents were placed into a Sarstedt tube. To remove the ethidium from the DNA sample, 5ml of butanol saturated with 1M NaCl was added and the solution was mixed the bottom layer was removed and this extraction step was repeated by this method 5-7 times, or until their was no pinkish tinge to the solution. To precipitate the plasmid, 1ml of TE buffer was added, along with 0.36 volumes of 7.5M NH_4OAc and 2.5 volumes 100% ethanol. After leaving at room temperature for 30 minutes, the mixture was spun in 50ml centrifuge tubes at 9,000rpm for 15 minutes using a SS-34 rotor (Sorvall) at room temperature. The pellet was washed in 70% ethanol, air-dried, and then redissolved in 250-500µl of distilled water and stored at -20^{0}C. To verify maxi prep purity, 1-2ml of the maxipreped sample are run down a 1% agarose gel to make sure there is no RNA smear at the bottom of the gel. If there is RNA contamination the maxiprep has to be treated with RNase A and reprecipitated to remove the RNase A which interferes with cell transfections. Plasmid DNA is quantified by reading absorbency at 260nm and 280nm using a DU 640B spectrophotometer (Beckman).

2.6.4 Qiagen

For preparation of large quantities of high-copy-number plasmids, QIAfilter Maxi kit and protocol was supplied by Qiagen. An individual positive colony was grown in 30 ml sterile glass tubes with 2 ml of antibiotic inoculated LB. The seed cultures were grown for 6 hours while shaking and were then used to inoculate an overnight 500ml culture. In the morning the cells were pelleted by centrifugation at 5,000rpm for 10 minutes at room temperature in 500ml centrifuge tubes. The supernatant was removed and the pellet was

resuspended completely in 10ml of buffer P1 (supplied). Then 10ml of buffer P2 (supplied) was added, and mixed gently into solution, followed by a 5 minute incubation. Finally 10ml of chilled buffer P3 (supplied) was added and the lysate was transferred directly to the barrel of the QIAfilter Maxi cartridge and incubated at room temperature for 10 minutes. A precipitate containing proteins, genomic DNA, and detergent forms during this time. Meanwhile a Qiagen-tip 500 was equilibrated with QBT buffer (supplied) and the filtered lysate from the QIAfilter Maxi is added directly and allowed to enter the resin by gravity flow. The column is then washed with two times 30 mls of Buffer QC (supplied), and then eluted after addition of 15ml of buffer QF. At room temperature 10.5 ml of isopropanol was added to the elution volume and the solution was centrifuged at 12,000rpm for 30 minutes at 4^0C. The supernatant was removed and the pellet was washed in 70% ethanol (5mls) and then centrifuged again at 12,000rpm for 10 minutes. The pellet was dried for approximately 5 minutes, and redissolved in 250-500µl of distilled water.

2.6.5 Quantification of Nucleic Acid

DNA was quantified roughly and assessed quickly for quality by running 1-5µl of diluted minipreped or maxipreped DNA down a 1% agarose gel plated on a cover slip. It was quantified more accurately using a DU-62 or a DU-640B, Beckman spectrophotometer set at 260nm. At 260nm one density unit was representative of 50µg/ml of double stranded DNA (**Maniatis *et al.*, 1982**).

2.7 Nucleic Acid Handling &Manipulation

2.7.1 Phenol Chloroform Extraction

Phenol was equilibrated to pH 7.5 with 1M Tris. 0.2% β-mercaptoethanol, 0.1% hydroxyquinoline, and stored in an equal volume of chloroform. Addition of phenol/chloroform removed proteins and/or salts from DNA solutions. After addition the samples were thoroughly mixed by vortexing and then the mix was centrifuged at 8000g-14000g for 10 minutes. The upper aqueous DNA containing layer was removed, leaving behind the proteinaceous interphase and phenol layers.

2.7.2 Ethanol Precipitation of Nucleic Acid

To precipitate DNA, 0.15M of NaCl, 2.5 volumes of 100% ethanol, and 5ul linear polyacrylamide (a precipitation aid that also allows for the visualisation of DNA) were add to sample tubes. The precipitation reaction was left overnight at -20^{0}C or at the very minimum for 3 hours before centrifuging the sample for 10 minutes at 14000g. The supernatant was then removed the supernatant and the pellet was then washed with 70% ethanol. After air-drying the pellet was resuspended in RNase A distilled water before quantification.

2.8 DNA Modification

2.8.1 Restriction Enzyme Digestion

To digest DNA fragments at specific sequences typically 5-10 units of restriction enzymes were added to a digestion reaction for every 200ng to 500ng of DNA present (Gibco BRL, Promega, Boehringer Mannheim). Whenever possible the conditions and buffers used for each of the enzymes were the ones the manufacturers determined to produce the best yields. Digestion reactions were normally incubated from 1-2 hours at 37^{0}C.

2.8.2 Shrimp Alkaline Phosphatase Dephosphorylation Reaction

To remove the terminal phosphate from the 5'-end group of a DNA strand 5 units of shrimp alkaline phosphatase (SAP) (U.S. Biochemicals) was added to the dephosphorylation reaction per microgram of DNA present. The reaction was carried out for 20-30 minutes with supplied buffer after which it was heat terminated for 10 minutes in a water bath kept at a constant temperature of 65^{0}C.

2.8.3 Ligation

In ligation reactions adjacent 3'-hydroxyl group and the 5'phosphate termini between vector and DNA insert strands were catalysed to form a linking phosphodiester bond. Typically 1 unit of enzyme catalyst, T4 DNA ligase (Boehringer Mannheim), 1x ligase reaction buffer (Boehringer Mannheim), and 1mM ATP was used in a 20 µl reaction vessel. The vector fragment was

dephosphorylated using Shrimp Alkaline Phosphatase (SAP) (Amersham), so that the vector could not religate to itself (for details see above section). Melted DNA/agarose or chip-dialysis retrieved DNA were added to the ligation reaction in several fold excess of the vector DNA, although the DNA/agarose addition did not exceed 1/5 of the total reaction volume. The reaction proceeded best if left overnight kept at temperatures of 15-25^{0}C and then heat terminated in the morning by heating to 72^{0}C for 5 minutes. Ligation products were directly transformed into bacteria.

2.8.4 Klenow Reaction

The Klenow fragment of the E.Coli DNA polymerase I makes 5' overhanging fragments into blunt ends. In a 25µl volume 1µg DNA was added to 2mM dNTPs, 10x nick translation buffer, 2 units Klenow fragment, and dH_2O. The mix was incubated for 15-20 minutes at 22^{0}C and then heated for 5 minutes to deactivate the enzyme.

2.9 Gel Electrophoresis of Nucleic Acids

2.9.1 DNA Agarose Gel Electrophoresis

Agarose gels for DNA fragment electrophoresis steps were prepared as follows. In 1 x TAE buffer 1-2%w/v agarose (SeaKem LE, SeaPlaque low melting point agarose, or MetaPhor high resolution agarose, FMC BioProducts, supplied by Flowgen Instruments Ltd.) was added and dissolved by heating in a microwave oven. To visualise smaller DNA fragments the higher percent agarose gels were used. When the agarose cooled yet was still in liquid form, 0.1 µg/ml ethidium bromide was added so that DNA strands run down the gel could be visualized under UV light (254nm). Using Teflon well combs to create lanes, gels were cast on 5cm x 7cm glass slides. Gels set on the slides due to the surface tension holding the liquid within the perimeters of the slide edge. Larger gels were made with 100ml of 1% agarose containing 0.1 mg/ml ethidium bromide in a gel caster from Gibco BRL to be run in an 11 by 14cm model H5 electrophoresis tank (Gibco BRL). The gels were run exclusively for the purposes of DNA lane chip-isolation and dialysis. Gels were typically electrophoresed at 55-100 volts in the small apparatus and 150 volts in the larger apparatus in 1xTAE buffer. Low melting temperature gels were always run below 65 volts so that

they would not melt. *Hind*III/*Eco*RI-digested bacteriophage Lambda DNA markers or supplied 0.1-12 kb markers (Ingenius MBM-005-100) were run next to experimental lanes. Important gels were photographed using Polaroid 667 land film in a Polaroid DS-34 hand-held direct screen camera while gels were UV illuminated at 254nm on a FotoPrepI (FotoDyne) transilluminator.

2.9.2 DNA Removal from Agarose Gels

Following DNA electrophoresis in ethidium containing gels on the large Gibco BRL Model H5 tank, the DNA was visualized by ultraviolet illumination at a preparatory wavelength of 300-360nm. In this way the DNA about to be extracted from the gel would not have been exposed to intense UV damage, as it is intended for DNA manipulation experiments. The band of interest is cut from the gel with a scalpel blade and scooped into an eppendorf. The gel chip is now ready for DNA extraction by the dialysis method.

Alternatively the DNA is electrophoreses onto a glass slip low-melting temperature agarose gel. UV examination at 300-360nm allows scalpel removal of the band of interest. The LM chip is scooped into an eppendorf and 5µl of TE buffer is added. The mixture is heated for 7-10 minutes at 65^0C to dissolve the LM chip in the buffer. Direct utilisation of this solution in ligation reactions is typical, as long as the total agarose concentration does not exceed 0.2% of the final reaction volume.

2.10 DNA Synthesis

The oligodeoxynucleotides mentioned in the results and discussion sections were synthesized by the Division of Biochemistry and Molecular Biology, Glasgow University. Care was taken to ensure oligodeoxynucleotide design did not introduce secondary structure and contained high G and C residue numbers for optimal template binding.

2.11 DNA Purification

2.11.1 Electroelution

Dialysis tubing was cut into approximately 10cm strips and pre-treated in 2% $NaHCO_3$ was stored in 50% ethanol at 4^0C. Gloves were worn whenever the

dialysis tubing was handled to prevent contamination. Prior to use, the dialysis tubing was boiled in dH_20 for 2-3 minutes, removing residual ethanol. The tubing was then clamped at one end and the inside of the tube was washed 2-3 times in fresh 1 x TAE buffer (no ethidium bromide). Meanwhile, the band with the PCR product of interest was isolated and cut carefully from a 1% agarose gel. The chip of agarose was placed long-ways in the dialysis tube and immersed in 2-3ml of 1 X TAE buffer. The open end of the dialysis tube was clamped so that the tube was not too taut, however, no air bubbles where left inside. In the electrophoresis tank, the dialysis tubing was placed long ways with the chip closest to the negative electrode end of the tube and was electrophoresed at 100 volts for 1 hour. Before discontinuing electrophoresis the electrodes were switched for 30 seconds at 100 volts, so that the DNA exiting the agarose chip was not propelled so far as to lodge in the dialysis tubing itself. The DNA containing 2-3ml buffer inside the tubing was removed.

2.11.2 Purification

Using a 5ml syringe (Beckton Dickinson) 1ml of high salt solution (Appendix 1) was taken up. The 21G Microlance-3^{TM} (Beckton Dickinson) needle head on the syringe was then replaced with a elutip-D column (Schleicher & Schuell) and the high salt was directed rapidly through the column. Using a fresh syringe 5ml of the low salt solution (Appendix 1) was taken up, and again the needle head was replaced with the column, so that the low salt solution could be rapidly pushed through to equilibrate it for binding DNA. The dialysis/DNA was pipetted into the back of the syringe column and was allowed to proceed dropwise through the column. The column was then washed with 3.5ml of low salt solution, again allowing it to proceed dropwise through the column. To elute the DNA, 800µl of high salt solution was allowed to pass through the column and the elution was collected in 2 eppendorfs, each with approximately 400ml. To precipitate the DNA 1ml of 100% ethanol was added to each tube and stored at -20^{0}C overnight. The next day the DNA was spun down

2.12 Construction Of Chimeric Receptor:

A chimeric receptor was made by fusing the extracellular domains of FcγRI to the intracellular domains of the T-cell associated gamma chain (Andre's

clone), the II subunit of FcγRII (George's clone), and the zeta chain subunit (Bryony's clone) (described in results section).

2.12.1 FcγRI-ζ Mutant

A fusion protein was constructed consisting of the FcγRI extracellular region attached to the transmembrane and cytoplasmic regions of the ζ chain of the T cell receptor. To introduce a 3' end *Ecl*XI site, as mentioned above, the ζ chain was PCRed up with restriction sites engineered into its end primers. The subsequent ζ chain sequence allowed for the easy subcloning of its transmembrane and cytosolic regions into the CDM vector already containing the extracellular FcgRI region (see results).

2.12.2 FcγRI-γ Mutant

Gamma chain was amplified from its pSVL plasmid using the forward primer (5'-GCCCTGGGGGATCCTCAGCTC-3') and the reverse primer (5'-AAGAATGCGGCCGCATCTATT-3'). The underlined sections of the primers are regions where BamHI and NotI restriction sites were introduced respectively. The BamHI site is at the γ-chain extracellular and transmembrane junction and the NotI site is just downstream of the stop codon. The resulting fusion protein has a FcγRI extracellular domain and γ chain transmembrane and cytoplasmic domains.

2.12.3 FcγRI-II Mutant

In the case of FcγRI-II, a similar approach was used. However, instead of a BamHI site at the extracellular/transmembrane junction a BglII site was engineered into the cDNA encoding FcγRIIa. This was because FcγRIIa has many BamHI sites that would interfere with the cloning strategy mentioned above. The sticky ends produced from BglII digestion allowed ligation of the fragment into the BamHI site.

2.13 Supplied cDNAs

The cDNA for FcγRIIa was a gift from Brian Seed (Department of Molecular Biology, Massachusetts General Hospital) and the cDNA for the γ chain was kindly provided by Jean Pierre Kinet (Harvard Medical School). Tyrosine

kinases used to test the relative phagocytic ability of the mentioned clones were obtained from various sources. mSyk was a gift from Martin Turner of Mill Hill, London, and mLyn and hFyn were gifts from Victor Tybulewicz of Mill Hill, London.

2.14 Sequencing

Sequencing of mutants was done by the Department of Biochemistry and Molecular Biology, Glasgow University, sequencing service using a the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq®DNA Polymerase, FS (Perkin Elmer). The reactions were carried out on a DNA Thermal Cycler Model 480 (Perkin Elmer).

2.15 Polymerase Chain Reaction (PCR)

PCR was used strategically to amplify specific sequences from a human library. Sequence oligonucleotide primers were designed with specific mismatched end base pairs that introduced restriction enzyme sites at the end of the amplified product, allowing for subsequent cloning attempts. Oligonucleotide primers were synthesised by the Glasgow University Biochemistry Department, and commercially by Oswell, using Applied Biosystems (Perkins Elmer) oligonucleotide synthesisers. PCR reactions were set up in reaction volumes of 50, 100, or 150µl in 750µl thin-walled eppendorf tubes. Reaction conditions varied depending on the DNA polymerase enzyme used. In reactions using 0.5 units of Dynazyme (Flowgen), 100-800ng linear DNA template, 10-100pmol of each oligonucleotide primer, 25mM dNTPs, 100mM DTT, 100mM $MgCl_2$, (found in the appropriate amount of 10x Dynazyme reaction buffer, Flowgen), and dH_20 to bring the reaction volume to the desired level.

For the Promega Taq PCR reaction, 50ng of template DNA was used. 50-100pmol of each primer was added, along with 10x reaction buffer, 1.5mM $MgCl_2$, 100µM dNTP's, 0.50 units Promega Taq, and dH_2O to a final concentration 100µls.

The reactions were overlaid with 50-150µl of mineral oil (Sigma) and tubes were placed in the RoboCycler™ 40 (Stratagene). The temperature cycling applied to tubes varied considerable. The annealing temperature in particular varied according to the oligonucleotide primer used and was typically 5-10^0C below melting temperature. An annealing temperature set

too low could allow for non-specific regions being amplified by the primer, and annealing temperatures too high may impede amplification of the desired product. The correct annealing temperature was calculated using the following equation published in the 1982 Maniatis Manual. For each percentage of mismatched nucleotide per primer a single ^{0}C was subtracted from the calculated melting temperature.

Melting Temperature = 69.3 + 0.41 (%G + C) - 650/L
(where L = length of the oligonucleotide primer)

Once in the Robocycler^{TM} 40, the first step was a high heat cycle that dissociated double stranded template: 3 minutes at 94^{0}C. A 30-40 repeat cycle followed with 3 variable steps which typically, in my case, included a 55^{0}C, 1 minute annealing step, a 72^{0}C, 2 minute DNA extension step, and a 94^{0}C, 45 second dissociation step. The cycle ended with a 45^{0}C, 5 minute final annealing step and a 72^{0}C, 20 minute final extension step. In the Promega Taq reaction the annealing step was split into a 5 cycles completed at 45^{0}C and 25 cycles completed at 55^{0}C.

Ten microliters of the PCR product was electrophoresed on a glass slide 1% agarose gel to check for PCR product. If the correct fragment was amplified, the rest of the PCR mix was ethanol precipitated to remove PCR buffer and concentrate the desired DNA fragments. Typically the reaction was made up to a volume of 200µl with dH_20 to which was added 1/10th volume 3.0M sodium acetate and 2 volumes 100% ethanol. Often the precipitation was left overnight at -20^{0}C and in the morning spun for 10 minutes at 12,000rpm in a microcentrifuge. The supernatant was discarded and the pellet was washed in 70% ethanol, respun for 5 minutes, the supernatant was again decanted off the pellet, which was finally left to air-dry. The pellet was finally resuspended in 10µl of TE buffer.

2.16 Reverse Transcriptase PCR (RT-PCR)

To isolate cDNA for zeta chain specific sequences RT-PCR was done on messenger RNA isolated from Jurkat T cells. Further amplification of the cDNA was tried using an enzyme from Thermus Thermophilus, Tth Polymerase (Promega Corp.). The enzyme catalyses DNA primer hybridisation to RNA template and subsequent nucleotide polymerisation in the 5'-3' direction. The reaction mixture was made as suggested by the

manufacturers of Tth polymerase. Eight microliters of RNA, as determined by X, 1mM $MnCl_2$. 0.2 mM of each dATP, dCTP, dTTP, and dGTP, 1 x Tth buffer (supplied), x pmol of RT-reverse primer, and x units of Tth polymerase were added to a thin walled 0.5ml PCR tube. The final volume of the reaction vessel was 20µl. For 20 minutes the reverse transcriptase reaction was allowed to be carried out at 70^0C. To arrest the reaction, EGTA containing chelate buffer (supplied) was added to tubes to remove Mn^{+2} and tubes were stored on ice for 5 minutes.

Tubes were then increased to a 100µl total volume with DEPC treated water, 2.5mM $MgCl_2$, x pmol of forward primer. An overlay of mineral oil was added to protect the PCR reaction from evaporation, and the tube was replaced in the Robocycler PCR machine (Stratagene) complete a DNA amplification and polymerisation cycling reaction. After cDNA denaturing step of 5 minute incubation at 95^0C the reaction had repeat cycling through a 1.5 minute, 92^0C denaturing step, a 1 minute, 55^0C, primer annealing step, and a 2 minute 70^0C nucleotide elongation step. This was repeated 30 times with a final 10 minute, 70^0C, elongation step at the finish of the cycle. RNase A was added to 10 and then 20 microliters of the reaction mixture which was then gel electrophoresed to assess RT-PCR success.

2.17 Northern Blot

Prior to gel electrophoresis RNA was denatured in a reaction mixture containing 1 x RNA electrophoresis buffer, 10% deionising formamide, and 10% formaldehyde. After 15 minutes heating at 65^0C, RNA samples in this mixture were considered to be denatured. The denatured RNA(approx. 40 µg per lane) was electrophoresed for 2-3 hours at 100 volts on a 1% agarose gel (15cm x 10cm x 1cm) containing 1 x RNA electrophoresis buffer, 25µg/ml ethidium bromide, 15% formaldehyde, and 0.1M iodoacetamide. The gel was then assessed under ultraviolet light at 254nm.

2.18 Transient Transfection

One day prior to transfection 100mm disposable polystyrene (Costar®) plates of COS-7 cells were trypsin digested and split into fresh plates at 20-30% confluency. These plates were left overnight and were typically at about 50% confluency, 4×10^7 COS-7 cells per plate, on the day of intended transfection.

On transfection day NU/100μM chloroquine medium (Appendix 1) was initially warmed to 37^{0}C in a water bath. About 5-10μg of each cDNA plasmid to be transfected was mixed with a 250μl of TE buffer and 180μl of DEAE-dextran (10mg/ml) in PBS. The 10% calf medium was aspirated off the COS-7 cells and approximately 5ml of the NU/chloroquine medium was added to each plate. In a dropwise fashion the cDNA mixture was added to each relevant plate and then swirled to provide a fairly uniform cDNA covering for the COS-7 cells. Plates were incubated at 37^{0}C for 3-4 hours, or until translucent bubbles appeared inside the vacuoles of the COS-7 cells. To catch the mixture before excessive cell death at this stage the NU/chloroquine solution is removed, the cells are shocked for 2 minutes in 5ml 1 x PBS with 10% DMSO. The cells are then washed 2 times in 5ml 1 x PBS lacking DMSO and reimmersed in 5ml of the standard 10% DMEM calf medium. The cells are incubated overnight at 37^{0}C and then are trypsin/split 6 ways onto fresh six well plates (Costar®). Phagocytosis assays were carried out 48-72 hours later when expression levels of the transfected cDNAs was at a maximum level.

2.19 Determination of Phagocytosis of Opsonized Sheep Red Blood Cells Using the Myeloperoxidase Method

Sheep red blood cells were stored a 4^{0}C in 50%v/v Alsever' solution (SAPU). One ml of the cells were removed and centrifuged at 3,000rpm for 2 minutes (Sorvall® MC12V microcentrifuge). The supernatant was removed and the cells were washed 3 times in 1 x PBS-EDTA (2mM). The cells were resuspended in 1 ml of 1 x PBS-2% BSA to which was added a subagglutinating of polyclonal rabbit antiserum which opsonized the SRBC's for internalization via Fc receptors. The SRBC's were thus left to incubate at 4^{0}C for 120 minutes, after which excess antibody was removed by 3 repeat washes in ice cold 1 x PBS-2% BSA, centrifugation at 3,000rpm for 2 minutes (Sorvall® MC12V microcentrifuge), and supernatant removal. The receptors being expressed were resuspended in 10ml of 10% Calf Serum DMEM (Appendix 1). Meanwhile, the medium covering the six well plate (Costar®) of the transfected COS 7 cells was aspirated and the cells were washed once with 1 x PBS and then overlaid with 1.5 ml of the SRBC suspension. The plates were left to incubate at 37^{0}C with 6.5% CO_2 levels for 2-4 hours.

After the incubation period excess SRBC were washed from the surface of all plates by repeat washing in 1 x PBS. Wells used to determine rosetting

were left for fixing at this point, while phagocytosis wells were subjected to a brief hypotonic shock (2-3ml of 1 x PBS pH 2.5 diluted to 1:150) to burst all SRBC that had not been internalized. A wash of 5ml 1 x PBS pH 2.5 which was immediately aspirated off and replaced by a 1 x PBS pH 7.4 wash, removed the residual brown staining left by burst SRBC's. The COS-7 cells were then left for 15 to 30 minutes in fixing solution containing 0.2M phosphate buffer (pH 7.4) and 0.5% gluteraldehyde.

At this point the cells were stained by the myeloperoxidase method for light microscopy visualisation (Appendix 1)**(Henson *et al.*, 1978; Whyte *et al.*, 1993)**. The staining solution was removed from the wells and Immu-mount (Shandon) was added dropwise to each well, while glass slips were dropped in place as a covering. In this way successful phagocytosis assays could be kept in storage.

The efficiency of phagocytosis is expressed as a phagocytosis index (PI). The PI is the multiplication of the mean number of internalised SRBCs per positive COS-7 cell (+/- SEM) and the percent positive cells on a transfection plate (a positive cell having engulfed at least one SRBC).

2.20 Protocols Leading to Western Blotting

2.20.1 Cell Pretreatment

U937 cells were differentiated into either FcγRI or FcγRII expressing cells. Confluent flasks of U937 cells were harvested into a Sarstedt tube and centrifuged for 3 minutes at 3,000 rpm in a Sorvall RT6000B benchtop centrifuge. The supernatant was removed and the cells were resuspended in fresh RPMI medium and divided in even aliquots between 3 flat bottom flasks (Costar®). The first flask was the control. The second flask was treated with 1mM dibutyl cyclic AMP (DBCA) and left incubating for 48 hours. The third flask was left for 24 hours like the control. Then the cells were respun and 1/2 the original media amount was added to the cells. The cells were then treated with X interferon gamma (IFN-γ) for 3 hours at which point the remaining 1/2 quantity of RPMI medium was added and the cells were left for 24 hours. All three flat bottom flasks were left with loose tops at 37^0C.

The U937 cells were harvested into a Sarstedt tube and centrifuged for 3 minutes at 3,000 rpm in a Sorvall RT6000B benchtop centrifuge. Control and experimental cells were each resuspended in cold 500 µl Hepes RPMI buffer. For the standard experiment 5 µl of IgG polyclonal antibody at 20µg/ml

(Meradex) was mixed into the samples and left for 30 minutes on ice. Cross-linking secondary antibody, at a standard 100µg concentration, was added and the cells were divided evenly between 1.5 ml eppendorfs. Over a time course that varied from zero minutes to 45 minutes, the cells were heated to active temperature of 37^0C on a heat pad, and then returned immediately to ice. The heating allowed for cross-linking to occur and phagocytosis to be initiated. The relevant protein involved in this process should become phosphorylated.

2.20.2 U937 Cell Lysis

One millilitre of ice cold PBS was added 2 times to the cells to wash them of excess primary and secondary antibodies. The cells were then spun for 3 minutes at 3,000 rpm in a Sorvall RT6000B and the supernatant was discarded. The cells were resuspended in 1ml RIPA lysis buffer per $1x10^7$ cells (as described in the U937 quantification methods section.) The cells were solubilised in a tumbling shaker (LabQuaker®, LabIndustries Inc.) at 4^0C for 2-3 hours. The samples were then microcentifuged at 12,000 rpm for 30 minutes at 4^0C and the supernatants were transferred to fresh tubes.

2.20.3 Immunoprecipitation

Agarose conjugated 4G10 Anti-Phosphotyrosine beads (TCS) were washed 3 times in RIPA lysis buffer and then spun for 10 minutes each time at 3,000 rpm in a Sorvall RT6000B. The beads were resuspended finally in a 1:1 ration with fresh RIPA Buffer. Approximately 10-20µl of the beads were added using cut pipette tips, to each cell lysate, and then rotated on the tumbling shaker (LabQuaker®, LabIndustries Inc.) at 4^0C overnight. The next day the samples were microcentrifuged at 3,000 rpm in a Sorvall RT6000B for 3 minutes and washed 3 times in 1 x PBS. Sample buffer was then added as described below.

2.20.4 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS Page gels were run on the Hoefer SE 600 vertical plates, each 16cm by 18cm. The apparatus was assembled using 1.5mm spacers to hold the plates apart. After the plates were clamped in this orientation an 8-10% polyacrylamide gel (the smaller the size of the desired protein the greater

percentage of acrylamide, was poured and overlay with butanol to prevent the edges of the gel from drying. The butanol was then drained and rinsed with dH_2O from the interface and a 2 inch in height layer of 4% stacking gel (Appendix 1) was added. A 15 well comb was then promptly inserted. Once dry the comb was removed and the gel apparatus was slightly rearranged so as to then fit in the running tank. The tank had two reservoirs encompassing both open ends of the gel. These reservoirs were filled with 1.5-2 liters running buffer (Appendix 1).

To load the samples each was resuspended in 50µl of SDS-Page sample buffer. The samples were boiled from 10 to 20 minutes to dissociate the phosphorylated tyrosine proteins from the sedimented agarose beads. The β-mercapto-ethanol in the sample buffer ensured the proteins to be run were in a reduced state. The samples were then briefly centrifuged and the supernatants were loaded into the wells of the gel using a Hamilton glass syringe (model 705N from Sigma). Care was taken not to load the beads into the wells of the gel. Markers were loaded alongside the protein samples. Wide range rainbow markers (6,500- 205,000 Daltons) from Sigma were used. Gels were run overnight at 60 volts with continuos magnetic stirring to keep the buffer circulating and cooling in the lower reservoir of the tank. Once the bromo-phenol blue dye reached the end of the gel, the electrophoresis was complete.

2.20.5 Western Blotting (BM Chemiluminescense)

Using the Hoefer® Transphor TE52X electro-transfer unit proteins were transferred from gels to nitrocellulose blotting paper. Pre-wetted Whatman® 3MM paper was placed on either side of the gel and nitrocellulose. Foam layers rested on the outside of the paper, and this sandwich was placed inside the transfer cassette. The transfer was carried out overnight at 60mA.

The nitrocellulose was blocked for 3 hours in 5% blocking (see Appendix 2). The membrane was then washed briefly in 1 xTBS and then a 3% blocking solution with specific primary antibody (concentrations described in results) was added. The filter was then incubated with gentle agitation (Belly Dancer® Scotlab) for 2-3 hours or overnight at 4^0C. The filter was then washed 5 x 5 minutes in 1 x TBS-Tween. A 1:1000 dilution of secondary antibody was then added to the membrane, using anti-mouse or anti-rabbit Horse Radish Peroxidase RPN 2108 (Boehringer Mannheim) depending on whether the primary antibody added was monoclonal or

polyclonal respectively. The filter was incubated with secondary for 30 minutes after which it was rinsed 5 x 5 minutes with TBS-Tween. The filter was then treated 1 minute with hand rotation submerged protein side up in detection solution supplied by the BM Chemiluminescence Western Blotting Kit (Boehringer Mannheim). The membrane was laid in between two layers of saran wrap and taped in an X-ray film cassette. In a dark room autoradiography film (Kodak) was placed in the cassette, and exposed to the luminous blot signal over various time points. The film was then developed in a X-omat XS-1 (Kodak).

2.21 Nitric Oxide Detecting Greiss Reaction

The Griess solution was made up by mixing Greiss solution A with Greiss solution B in a 1:1 v/v ratio. A standard curve was set up in rows A & B of a 96 well plate. To all of row B and row A, wells 4-12, 50µl of distilled water was added. Then 50µl of a 10µg/ml stock of $NaNO_2$ (145nmol/ml nitrite) was added to row A, wells 1-6. Doubly dilute down from row A, wells 4-6. The last three wells of row B were left with distilled water only.

At this stage 50µl of Greiss solution was added to the standard curve wells and to experimental wells each containing 50µl of sample. The plate was then left for 10 minutes and then read in an ELISA reader at 570nm with a 630nm reference filter (Dynatech Laboratories). The resulting standard curve is 145, 72.5, 36, 18, 9, 4.5, 2.3, and then 0.0 nmol/ml nitrite. The more nitrite in the solution, the more azo-compound resulting from the mix, and the greater the color change to pink. For experiment specifics see the results section.

Chapter 3

Results Part I

3.1 Introduction to Western Results

As discussed in the introduction, macrophages function as both antigen presenting cells and as cells capable of antigen dependent cellular cytotoxicity. Antigen internalisation is made possible by a number of specialised receptor components, FcγR's and their various associated subunits. Cross-linking of these FcγR's triggers antigen dependent cytotoxicity.

Firstly, these studies investigated the differences between signal transduction events within the cell for FcγRI + γ chain and FcγRII on U937 monocyte-like cells. The focus was to define the pattern of tyrosine kinase phosphorylation after FcγR cross-linking in differentiated and then undifferentiated monocytes.

Secondly, macrophages have different release response products following activation including superoxides, cytokines, and the vasodilator nitric oxide. In these studies it was hoped that specific Fc receptors could be linked to specific nitric oxide release responses in differentiated and undifferentiated U937 cells.

3.2 Evidence That IFNγ Treated Cells Show a Peak Increase in Syk Recruitment 5 Minutes Post-Activation (Figure 3.1.1)

In IFNγ treated U937 cells both FcγRI and γ-chain were upregulated. As described in detail in the material and methods section, hIgG polyclonal was added (standard amount) to the 4×10^6 cells per samples so that theoretically FcR would bind the antibody but at the non physiological temperature of 4^0C, so no cross-linking should have followed. A secondary cross-linking antibody was then added to selected samples. In the case of blot 3.1.1, sheep anti-human cross-linking antibody was added at 100μg concentration. The samples were heated to the physiologically active temperature of 37^0C over a time course of 1 minute, 5 minutes and finally 35 minutes. The temperature raise should increase the receptor movement on the cell surface, allowing

Figure 3.1.1

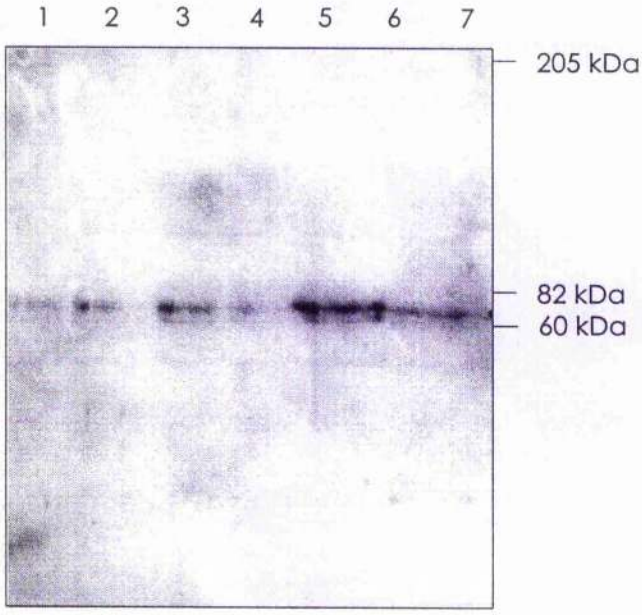


Figure 3.1.2

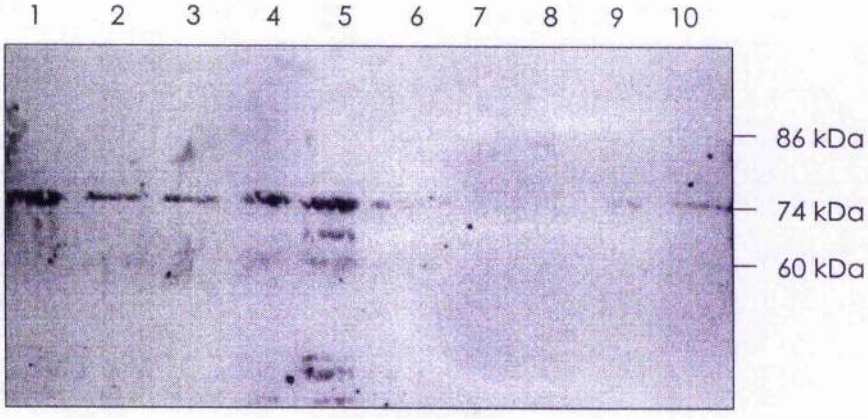


Figure 3.1.1 IFNγ treated U937 cells were activated, lysed, and then immunoprecipitated with anti-phosphotyrosine agarose beads (TCS). The phosphorylated proteins were dissociated from the beads and run down an 8% SDS-Page gel, and then transferred to nitrocellulose. The filter was probed for the presence of Syk (74kDa) with anti-syk antibody. **Lane 1** Marker. **Lane 2** Control lane. **Lane 3** hIgG polyclonal primary only. **Lane 4** hIgG polyclonal and sheep anti-human cross-linking antibody. **Lane 5** hIgG polyclonal and sheep anti-human cross-linking antibody heat activated for 1 minute. **Lane 6** Same as Lane 4 but heat activated for 5 minutes. **Lane 7** Same as Lane 4 but heat activated for 35 minutes.

Figure 3.1.2 IFNγ treated U937 cells were activated, lysed, and then immunoprecipitated with anti-phosphotyrosine agarose beads (TCS). The phosphorylated proteins were dissociated from the beads and run down an 8% SDS-Page gel, and then transferred to nitrocellulose. The filter was probed for the presence of Syk (74kDa) with anti-syk antibody. **Lane 10** Control. **Lane 9** hIgG polyclonal primary only. **Lane 8** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 1 minute. **Lane 7** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 3 minutes. **Lane 6** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 5 minutes. **Lane 5** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 7 minutes. **Lane 4** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 9 minutes. **Lane 3** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 12 minutes. **Lane 2** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 15 minutes. **Lane 1** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 30 minutes.

aggregation through cross-linking antibody contact. In the heated samples it was anticipated that there would be activation of cell signalling processes. The sample cells were then lysed and anti-PT agarose (TCS) was used to bind all phosphorylated proteins found in the lysate. These proteins were run down an 8% gel and transferred to a nitrocellulose membrane (Schliecher and Schuell). The blot was probed with anti-syk antibody at 100µg concentration (A generous gift from Dr. M. Harnett, Department of Immunology, Glasgow University).

In this successful pilot study it was found that phosphorylated Syk was found in cells 5 minutes at a peak post activation level which had declined slightly by the 35 minute mark. As the background was quite dark on this blot, the blot was stripped and reprobed, this time using 2µCi of an ^{125}I radiolabelled goat anti-rabbit antibody as secondary antibody. This was done as ECL, although easy to use, often has a high background signal, making the blots less defined compared to probings with radioactive antibody.

After a 1 hour incubation the blot was washed 3 x with TBS for 20 minutes each time. The nitrocellulose was laid out on a wet piece of Whatmann® 3MM and heat sealed in a polythene sheet (Salton). The filter was exposed 4 hours to a 20cm x 40cm imaging plate (type BAS-IIIs:Fuji). Using a Fuji 100 Phosphoimager (Imager) and the display package on an apple mac computer, the plate was scanned. The result was weak with band density not better than the original ECL blot. It was thought that perhaps the nitrocellulose used from Schliecher and Schuell was not designed for repetitive probing, and perhaps this was why so much of the signal was lost.

3.3 Further Evidence That IFNγ Treated Cells Show a Peak Increase in Syk Recruitment 5-7 Minutes Post-Activation (Figure 3.1.2)

To test further the pattern of Syk recruitment in IFNγ treated U937 cells the first experiment was repeated with a more extensive time course of secondary cross-linking over the critical time period. For blot 3.1.2, goat anti-human Fab specific secondary antibody (Serotech) was added at 100µg concentration. The samples were cross-linked at 37^0C for 1, 3, 5, 7, 9, 12, 15, and 30 minutes. The anti-Syk probed nitrocellulose was developed by ECL techniques. There was a significant increase in Syk recruitment in between 5 and 7 minutes post-activation and the Syk levels remained high even at the 30 minute post-activation time point.

3.4 Evidence That DBC Treated Cells Show an Immediate Increase in Syk Recruitment Following Activation (Figure 3.1.3)

Now that a preliminary pattern of Syk recruitment in IFNγ cells was established, tests were started to try and deduce an alternative pattern of TK recruitment in DBC treated cells. DBC treated U937 cells differentiate into a more macrophage-like cell upregulating FcγRII expression and down-regulating FcγRI and γ-chain. In the first test a blot from a previous experiment was re-wetted and reprobed with the anti-Syk antibody. The original gel was loaded with 4×10^6 cells in sample tubes with primary hIgG antibody and then adding goat anti-human Fab specific secondary antibody. The samples were placed at 37^0C over a time course of 1, 3, 5, 7, 9, 12, 15, and 30 minutes. The samples were lysed, immunoprecipitated with anti-PT agarose beads, and the proteins were run on an 8% gel. This time the proteins were transferred to a PVDF membrane (Flowgen) which is a durable membrane that allows for multiple reprobings. The membrane must be treated before use with a 5 minute soak in methanol and then a 5-10 minute submersion in the relevant transfer buffer.

In this reprobing experiment the PVDF membrane in question was stored after stripping at -20^0C surrounded on either side by wet Whatmann® 3MM paper and sealed in an polythene bag. When ready to use it was thawed at room temperature treated with methanol and transfer buffer as mentioned previously.

The blot was reprobed in a similar manner to the IFNγ blots 3.1.1 and 3.1.2. with anti Syk antibody and ECL developed. After a 3 minute exposure the signal at the appropriate molecular weight for Syk tyrosine kinase was observed. However, there are large areas of negative holes in the background signal over the whole area of the blot. It looks as if these areas were not hydrated properly after blot thawing and remained unable to bind primary and secondary antibody. In the next reprobing experiment and thereafter, after stripping the PVDF membrane it was dried at room temperature on dry Whatmann® 3MM paper. Then the blot was stored at room temperature in a dry folder until retreated and reprobed. This method prevented the blotchy appearance of the frozen and then thawed blot used in this experiment.

From the blot some information can be deduced. As can be seen, although it is difficult to read whether control U937 and primary antibody lanes have signal, it is obvious that DBC cells have Syk activation in cells as

Figure 3.1.3

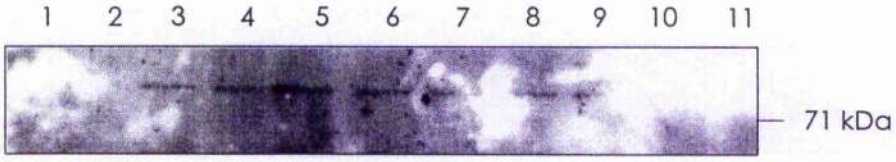


Figure 3.1.4

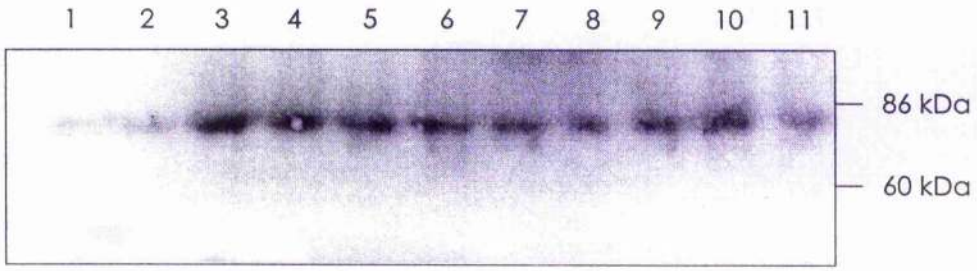


Figure 3.1.3 DBC treated U937 cells were activated, lysed, and then immunoprecipitated with anti-phosphotyrosine agarose beads (TCS). The phosphorylated proteins were dissociated from the beads and run down an 8% SDS-Page gel, and then transferred to nitrocellulose. The filter was probed for the presence of Syk (74kDa) with anti-syk antibody. **Lane 11** Control. **Lane 10** hIgG polyclonal primary only. **Lane 9** hIgG polyclonal and goat anti-human Fab specific secondary antibody. **Lane 8** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 1 minute. **Lane 7** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 3 minutes. **Lane 6** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 5 minutes. **Lane 5** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 7 minutes. **Lane 4** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 9 minutes. **Lane 3** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 12 minutes. **Lane 2** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 15 minutes. **Lane 1** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 30 minutes.

Figure 3.1.4 DBC treated U937 cells were activated, lysed, and then immunoprecipitated with anti-phosphotyrosine agarose beads (TCS). The phosphorylated proteins were dissociated from the beads and run down an 8% SDS-Page gel, and then transferred to nitrocellulose. The filter was probed for the presence of Syk (74kDa) with anti-syk antibody. **Lane 1** Control. **Lane 2** hIgG polyclonal primary only. **Lane 3** hIgG polyclonal and goat anti-human Fab specific secondary antibody. **Lane 4** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 1 minute. **Lane 5** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 3 minutes. **Lane 6** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 5 minutes. **Lane 7** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 7 minutes. **Lane 8** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 9 minutes. **Lane 9** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 12 minutes. **Lane 10** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 15 minutes. **Lane 11** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 30 minutes.

early as 1 minute post activation. Although there is immediate presence of signal post activation there may be a peak of activation at 9 minutes. However, because of the blotchy nature of this blot, a repeat of the pilot is necessary.

3.5 Further Evidence That DBC Treated Cells Show an Immediate Increase in Syk Recruitment Following Activation (Figure 3.1.4)

In the next experiment 1 x 10^7 cells were used per sample. DBC treated cells were treated identically to IFNγ treated cells in Figure 3.1.2. Interestingly, Syk signal can be detected as early on as after the addition of primary antibody. This should not have happened so early as precautions were taken to keep sample under physiological temperature between treatment with primary and then secondary cross-linking antibody. This may have happened because the ice it was stored in may have been slightly melted over the 30 minutes the other samples were treated. This lowering of temperature may have facilitated slight receptor activation via the FcγRII tail signalling domain that is an integral part of this receptor. In IFNγ treated cells receptor aggregation also brings into close proximity the γ-chain which is required by FcγRI for signalling.

In blot 3.1.4 the Syk signal is relatively uniform across all samples in the time course from 1 minute to 15 minutes, slightly tailing off at the 30 minute mark. This corroborates the 3.1.3 blot results that shows relatively uniform band densities indicating Syk activation almost immediately following receptor cross-linking (and in this case even before cross-linking in the primary antibody only lane).

Although in blot 3.1.4 the amount of Syk present seems relatively constant across each lane. This may be representative of anti-phosphotyrosine agarose beads being over saturated with protein in this experiment. The standard 10µl of beads were added to each sample which contained lysates of considerably more protein, since more cells were lysed in this experiment 1 x 10^7 cells, versus the standard 4 x 10^6 cells lysed in IFNγ samples. However, a low beads to protein ratio would not explain the DBC results from blot 3.1.3, which show a similar trend of immediate Syk recruitment post activation, and the standard 4 x 10^6 cells were used in this experiment. So in these preliminary experiments, while IFNγ cells show peak Syk activation 5-7 minutes post activation that is still high 30 minutes later,

DBC cells show an immediate increase in Syk recruitment following activation that is tailing off by the 30 minute mark.

3.6 Evidence That the Buffer's Protease Inhibitors Had Degraded, Hampering Further Experiments (Figure 3.1.5)

Although other blots were attempted to further confirm the results seen above,. several things complicated the other experiments. It was finally confirmed that the protease inhibitors in the RIPA buffer were no longer effectively preventing degradation of cellular proteins in the lysates. As can be seen in blot 3.1.5, this was originally a repeat of 3.1.1, run on a 12% gel instead of an 8% gel. The blot resembles one of proteins probed with anti-phosphotyrosine antibody, and not that of phosphorylated proteins probed specifically with anti-Syk.

Another test of the remaining anti-Syk polyclonal antibody in the lab (a kind gift from Dr. Maggie Harnett, Dept. of Immunology, Glasgow University) showed that the antibody itself had degraded (data not shown). For this reason a commercial Syk polyclonal antibody was ordered for future blot probes.

3.7 Lysates Probed with Anti-Phosphotyrosine Antibody Yielded a 110 kDa Band for the IFNγ Blot and Both 110 and 50 kDa Bands for the DBC Blot (Figure 3.1.6 and 3.1.7)

In a pilot test 4 x 10^6 cells per sample of both IFNγ and DBC cells were treated with polyclonal human IgG and then over a time course of 1, 3, 5, 7, 9, 12, 15, and then 30 minutes secondary goat anti-human antibody at 37^0C cross-linked the receptors on the cell surface. Cells were lysed as always, however, on this occasion the lysates themselves were spun for 30 minutes at 12 rpm and the supernatants were run down an 8% gel. The proteins were transferred onto PVDF membranes and probed with anti-phosphotyrosine antibody (TCS) at 100µg concentration to bring up all phosphorylated proteins present in the lysate. Although a smear of proteins was expected, strangely only several bands showed up. Perhaps the low number of cells allowed only greatly over-activated proteins to be apparent. The IFNγ blot yielded a single band of about 110kDa. Interestingly, while the DBC blot had bands at the same location as the IFNγ blot at 110kDa, it also had a second line of bands at around 50kDa. In the DBC filter the bands at 110kDa and 50kDa

Figure 3.1.5

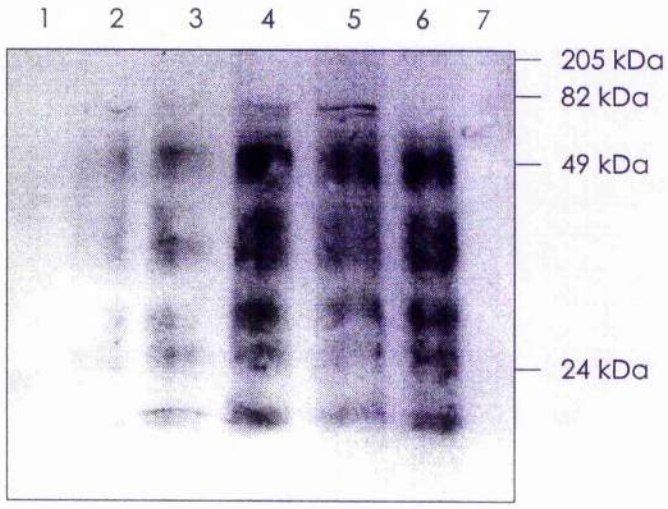


Figure 3.1.5 Originally a repeat of 3.1.1, run on a 12% gel instead of an 8% gel. Shown as an example of the effect of degraded protease inhibitors in the RIPA buffer no longer being able to prevent degradation of cellular proteins in the lysates.

Figure 3.1.6

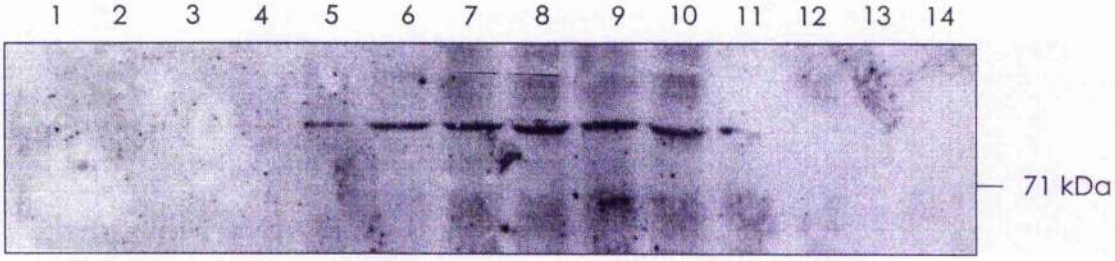


Figure 3.1.7

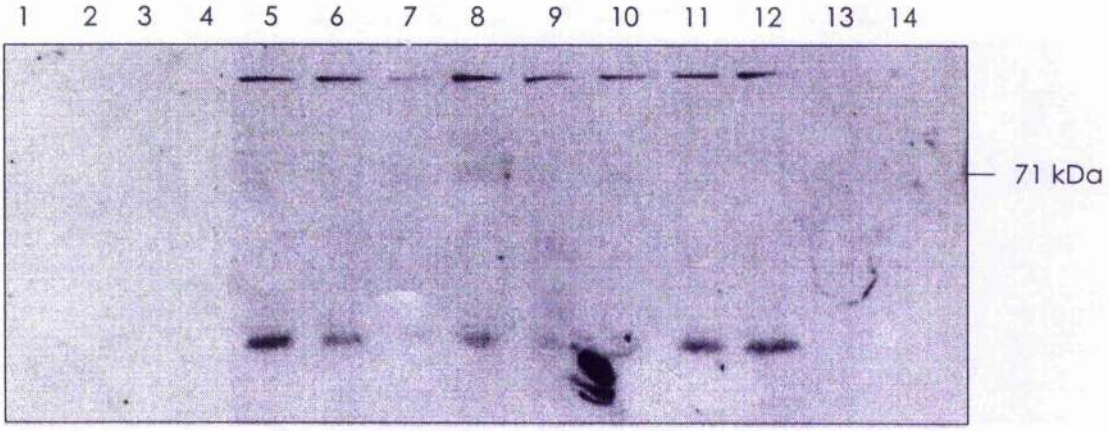


Figure 3.1.8

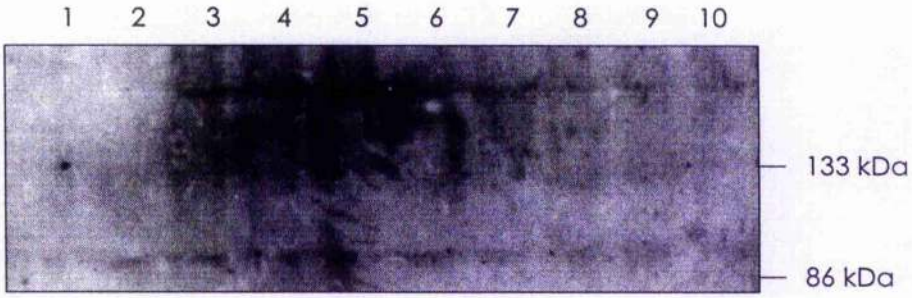


Figure 3.1.6 IFNγ treated U937 cells were activated, lysed, and then the lysates themselves were run down an 8% SDS-Page gel, and then transferred to nitrocellulose. The filter was probed for the presence of phosphorylated proteins using an anti-phosphotyrosine antibody. In Lanes 14-4, 4×10^6 cells were lysed per sample and 50µl were added per well. In Lanes 3-1, 1×10^6 cells were lysed per sample and 50µl were added per well. **Lane 14** Control. **Lane 13** hIgG polyclonal primary only. **Lane 12** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 1 minute. **Lane 11** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 3 minute. **Lane 10** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 5 minutes. **Lane 9** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 7 minutes. **Lane 8** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 9 minutes. **Lane 7** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 12 minutes. **Lane 6** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 15 minutes. **Lane 5** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 30 minutes. **Lane 4** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 30 minutes. **Lane 3** Control. **Lane 2** hIgG polyclonal primary only. **Lane 1** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 1 minute.

Figure 3.1.7 DBC treated U937 cells were activated, lysed, and then the lysates themselves were run down an 8% SDS-Page gel, and then transferred to nitrocellulose. The filter was probed for the presence of phosphorylated proteins using an anti-phosphotyrosine antibody. In Lanes 14-4, 4 x 10^6 cells were lysed per sample and 50µl were added per well. In Lanes 3-1, 1 x 10^6 cells were lysed per sample **Lane 14** Control. **Lane 13** hIgG polyclonal primary only. **Lane 12** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 1 minute. **Lane 11** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 3 minute. **Lane 10** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 5 minutes. **Lane 9** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 7 minutes. **Lane 8** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 9 minutes. **Lane 7** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 12 minutes. **Lane 6** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 15 minutes. **Lane 5** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 30 minutes. **Lane 4** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 30 minutes. **Lane 3** Control. **Lane 2** hIgG polyclonal primary only. **Lane 1** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 1 minute.

Figure 3.1.8 A general anti-src antibody was used to probe a lysate blot, prepared in the same manner as blot 3.1.7. **Lane 10** Control. **Lane 9** hIgG polyclonal primary only. **Lane 8** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 1 minute. **Lane 7** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 3 minutes. **Lane 6** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 5 minutes. **Lane 5** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 7 minutes. **Lane 4** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 9 minutes. **Lane 3** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 12 minutes. **Lane 2** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 15 minutes. **Lane 1** hIgG polyclonal and goat anti-human Fab specific secondary antibody heat activated for 30 minutes.

appeared at 1 minute and were approximately the same density and present in the lane corresponding to 30 minutes activation.

3.8 DBC Lysate Blot Probed with General Anti-Src Antibody (Figure 3.1.8)

To try and establish the identity of the bands from blots 3.1.6 and 3.1.7, and in particular the 50kDa band that was present in the DBC blot and not the IFNγ blot, a second experiment using just 4 x 10^6 cells per sample and following the lysis protocol used for blots 3.1.6 and 3.1.7 were probed with a general anti-src antibody (Dr. M.Harnett) at 100μg concentration and developed by ECL techniques (Figure 3.1.8). The IFNγ blot did not yield any visible bands, while the DBC blots yielded bands at approximately 150kDa and one set of bands at approximately 90kDa. However, neither of these DBC bands corresponded to the bands of phosphorylated proteins found on the original filter. The identity of the 110kDa bands on IFNγ and DBC blots, and the 50kDa band, found exclusively on the DBC blot, remains unknown.

3.9 Nitric Oxide Production

Comparisons were made of downstream activation events including nitric oxide production in differentiated and undifferentiated U937 monocyte-like cells.

3.9.1 Greiss Reaction

In conjunction with Professor E. Liew in the Department of Immunology, Glasgow University, the Greiss reaction was used to test for nitric oxide presence in the supernatant of treated U937 cells. Flasks of U937 cells were alternatively treated with either DBC for 48 hours, to upregulate FcγRI/II, or IFNγ for 24 hours, to upregulate FcγRI + γ-chain. The confluent cells were then harvested and spun. The old supernatant was removed and the cells from both flasks were each resuspended in 10ml of RPMI with 10% fetal calf serum. For each batch 2 ml was added to each of 5 wells in 6 well plate (one well remained empty). To both IFNγ and DBC plates the wells were treated as follows; well 1 was the control, well 2 contained primary antibody only, well 3 contained primary and secondary cross-linking antibody, well 4 contained 2 ml SRBCs, well 5 contained 2 mls SRBCs opsonized with

subagglutinating polyclonal rabbit antiserum. In this way, well 1 was a control, while wells 2 and 4 were controls for endocytosis and phagocytosis respectively. Wells 3 and 5 were endocytosis wells and phagocytosis wells respectively.

Plates were left incubating at 37^0C for 3 hours. At this stage 50ml of supernatant from each well was transferred to an Elisa plate and tested with Greiss solution (alpha-naphtyl-amine, sulphanilamide, and phosphoric acid) that reacts to NO_2 in solution (standard protocol). The Elisa reader set at 570nm showed no significant NO production.

3.9.2 Search for Nitric Oxide Synthase (hNOS)

Another method was used to try and detect nitric oxide production in activated monocytes. Again U937 cells were treated with either DBC for 48 hours, to upregulate FcγRI/II, or IFNγ for 24 hours, to upregulate FcγRI + γ-chain. The cells were spun and lysed with RIPA buffer as before and heated for 10 minutes at 37^0C. The cells were vortexed, and then heated for 10 minutes at 37^0C, and then 10 minutes at 50^0C. Once completely soluble and no longer sticky the samples were heated to 100^0C for 5 minutes to denature the proteins, which could be then stored at -80^0C.

A 7.5% acrylamide gel was run with 50µl per well of the DBC and IFNγ lysed U937. The proteins on the gel were transferred to a nitrocellulose gel and probed for 1 hour with a 1/10,000 dilution of polyclonal antibody to hNOSi (Merck) raised against C-terminal peptides. The secondary used was anti-rabbit horse radish peroxidase. Probing followed the standard protocol mentioned in the materials and methods. Using the BM Chemiluminescence kit detection solutions the blot was treated and then exposed to x-ray film. hNOS should appear as a band of approximately 130kDa. There was no positive result.

Since even the markers and positive control didn't appear on the film in the last experiment it was suggested that the old BM Chemiluminescence solutions may not have been working. The gel and blot was repeated as above and primary polyclonal antibody against hNOSi was incubated as before. The filter was this time blocked and 2µCi of an ^{125}I radiolabelled goat anti-rabbit antibody (Serotec) was added to the blocking solution. After a 1 hour incubation the blot was washed 3 x with TBS for 20 minutes each time. The nitrocellulose was laid only on a wet piece of Whatmann® 3MM and heat sealed in a polythene sheet (Salton). The filter was exposed 4 hours to a 20cm

x 40cm imaging plate (type BAS-IIIs:Fuji). Using a Fuji 100 Phosphoimager (Imager) and the display package on an Apple Mac computer, the plate was scanned. Again this experiment yielded a negative result.

Chapter 4

Results Part II

4.1 Introduction to Phagocytosis Results

The body of this work focused on the mechanism by which Fcγ receptors, mainly isotypes I and II, are able to recruit accessory molecules capable of activating soluble tyrosine kinases.

These results examine FcγR signalling in the heterologous cell system of COS cells (a simian kidney fibroblast cell line). A chimeric receptor was made by fusion of the extracellular region of FcγRI to the transmembrane and cytoplasmic tail of zeta chain. This chimeric receptor was then compared to other previously constructed mutants with altered signalling domains for its ability to phagocytose sheep red blood cells (SRBC) in a well established phagocytosis experiment developed in the lab.

4.2 CDM Vector

PCR primers were designed to isolate human zeta and mouse zeta chains from the human lac and mouse thymus cDNA libraries respectively. The PCR products were originally going to be ligated into the CDM vector, so primers had a forward *Hind*III and a reverse *Ecl* XI site engineered into them so they were compatible with an ideal CDM insertion site.

4.3 Dynazyme PCR of the Mouse Zeta Chain

Initially there was considerable difficulty in retrieving the desirable sequence from PCR (see standard protocol in material and methods). A variety of parameters were altered to try and achieve more ideal conditions.

The PCR parameters altered were as follows. In the original reaction both the mouse and human library had quite a high concentration at 800ng per reaction. This may have been a major obstacle hampering polymerase ability in the reaction solution. Initially the template DNA was also resuspended in TE buffer, as were the primers. In later reactions they were resuspended in dH_2O. The EDTA in the TE buffer may have affected the

Dynazyme polymerase and kept it from working properly, as Dynazyme is reportedly especially EDTA sensitive. The concentrations of primers added were increased from a very dilute 10pmol per reaction up to 100pmol. Neither primer amount was optimal. The final working primer concentration was 30pmol for both human and mouse zeta chain PCR. As Dynazyme came with a buffer containing optimal magnesium concentrations, this parameter was not altered, although it still could have been.

Much of the difficulty may have been due to the non-degenerate nature of the primers, with a considerable degree of mismatching. For each percentage of mismatched nucleotide per primer a single ^{0}C was subtracted from the calculated melting temperature.

Melting Temperature = 69.3 + 0.41 (%G + C) - 650/L

(where L = length of the oligonucleotide primer)

Although the ideal annealing temp was calculated to 61.7^{0}C-65.7^{0}C for human zeta primers and 61.7^{0}C-65.6^{0}C for mouse zeta primers, a range of annealing temperatures were tried from 35^{0}C-65^{0}C. A special PCR program, Touchdown PCR Strategy, was used to assay a single PCR reaction over a 8^{0}C range of the 42^{0}C-50^{0}C annealing temp over a single 40 cycle PCR program, with 5 cycles at each annealing temperature. This too was unsuccessful.

In one reaction with the above conditions a slightly visible band was observed for mouse zeta in the mouse thymus library. I used the same template from the reaction that "worked" and repeated the PCR using fresh primers and dNTPs and polymerase. No increase in product was observed. I repeated the original reaction increasing the cycles of the PCR from 30 to 40. Since Dynazyme is a fairly reliable polymerase it was hoped that the additional cycling would not introduce any sequence mismatches. The mouse zeta chain DNA of 565bp was successfully PCRed using the Dynazyme enzyme reaction conditions and a 55^{0}C annealing temperature (see Materials and Methods).

4.4 Mouse Zeta

After PCR retrieval the mouse zeta was ligated into SAP enzyme treated pBS instead of CDM via its engineered HindIII and EclXI end restriction sites. The ligation product was transformed into TG1 competent cells, however, after many repeated attempts and repeat ligations only blue colonies were recovered from this stage.

4.5 RT-PCR Retrieval of Human Zeta Chain

Jurkat p10 cells were lysed to recover T cell RNA using the Guathio-RNA extraction method. On the resulting two RNA samples, 260/280 spectrophotometer readings revealed 1.9, and 1.7 ratios respectively, both well within the range of high quality RNA. However, RT-PCR was unsuccessful after 3 attempts. A Northern was conducted on the 2 samples to further determine relative quality. The appropriate bands **(High and low subunit?)** were present and by all appearances the RNA remained undegraded.

4.6 Promega Taq PCR of the Human Zeta Chain

This PCR was accomplished using the Hpball human library. The libraries used were diluted so that in the reaction vessel 50ng of DNA was present (see Materials and Methods). The reactions were repeated in triplicate. Also for each library, Hlac and Hpball, both Dynazyme and Promega Taq were used under the new reaction conditions. At the annealing phase 5 cycles were completed at 45^0C and 25 at 55^0C. Although there were 3 positive lanes with the desired 574 bp product in the Hpball template assays, there were none in the Hlac experimental lanes. Perhaps there was considerable degradation in the Hlac library, which would explain the considerable difficulty found when previously working exclusively with this library.

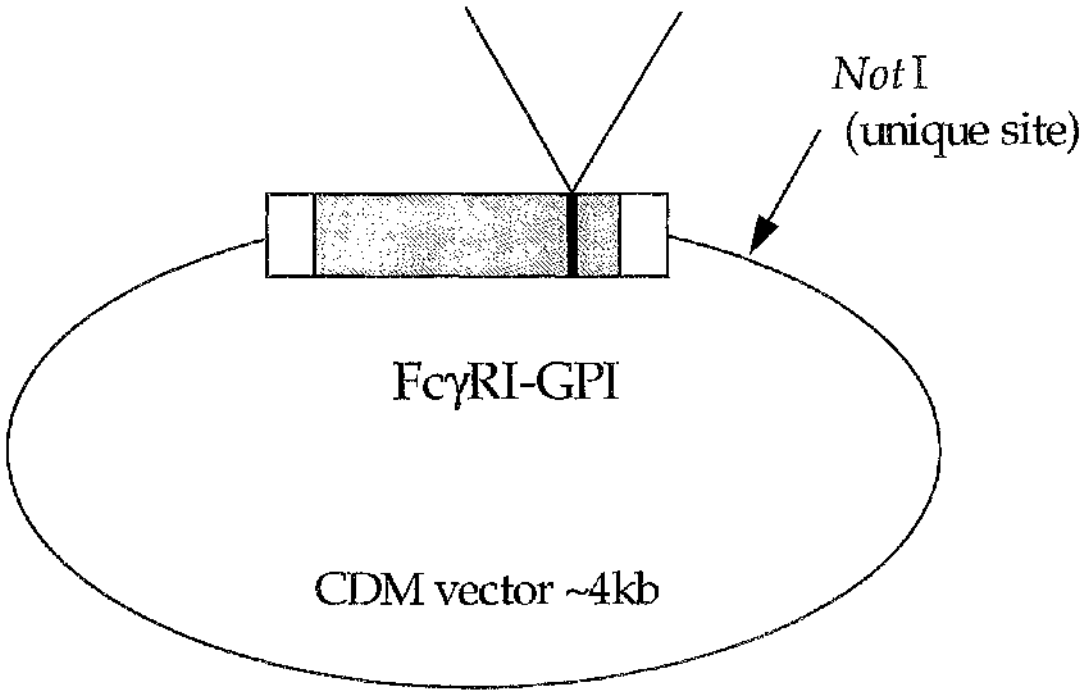
4.7 Novel FcγRI-ζ Mutant

A fusion protein was constructed consisting of the FcγRI extracellular region attached to the transmembrane and cytoplasmic regions of the ζ chain of the T cell receptor. To introduce a 3' end *Ecl*XI site, as mentioned above, the ζ chain was PCRed up with restriction sites engineered into its end primers. The subsequent ζ chain sequence allowed for the easy subcloning of its transmembrane and cytosolic regions into the CDM vector already containing the extracellular FcγRI region.

The human zeta chain cDNA was obtained by PCR using primers to sequences in the untranslated regions flanking the open reading frame. The primers introduced a 5' end *Hind*III restriction site and a 3' end *Ecl*XI restriction site. The PCR product was inserted into the pTag cloning vector for amplification (Invitrogen). Interestingly the human ζ chain possesses a

H*M D P

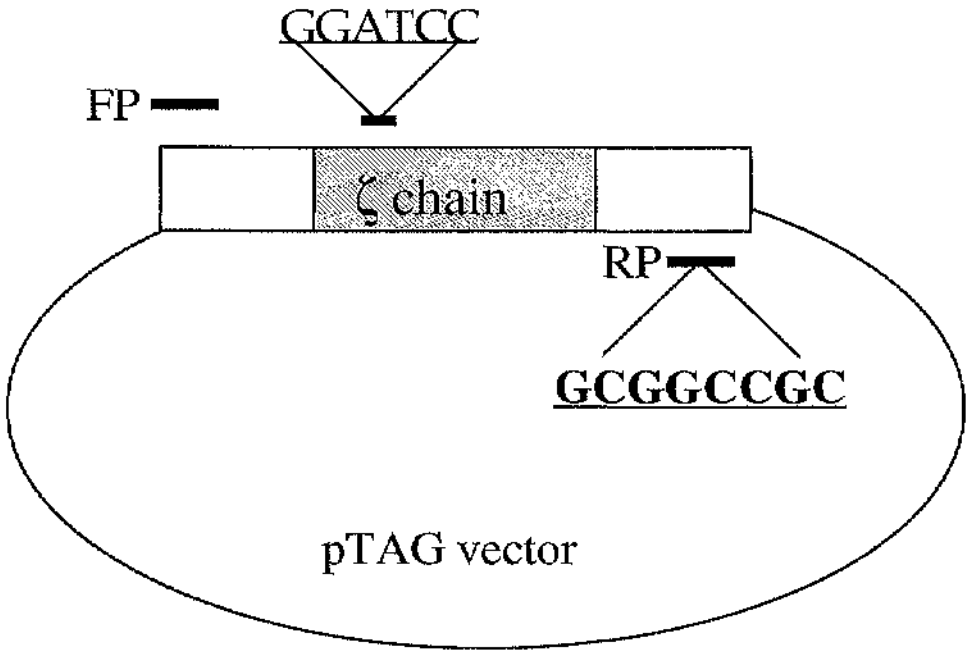
CATATGGATCCT



H*	Final amino acid of FcγRI extracellular domain
GGATCC	*BamHI* site
MDP	Amino acids created by cloning cassette
	Open reading frame of FcγRI-GPI

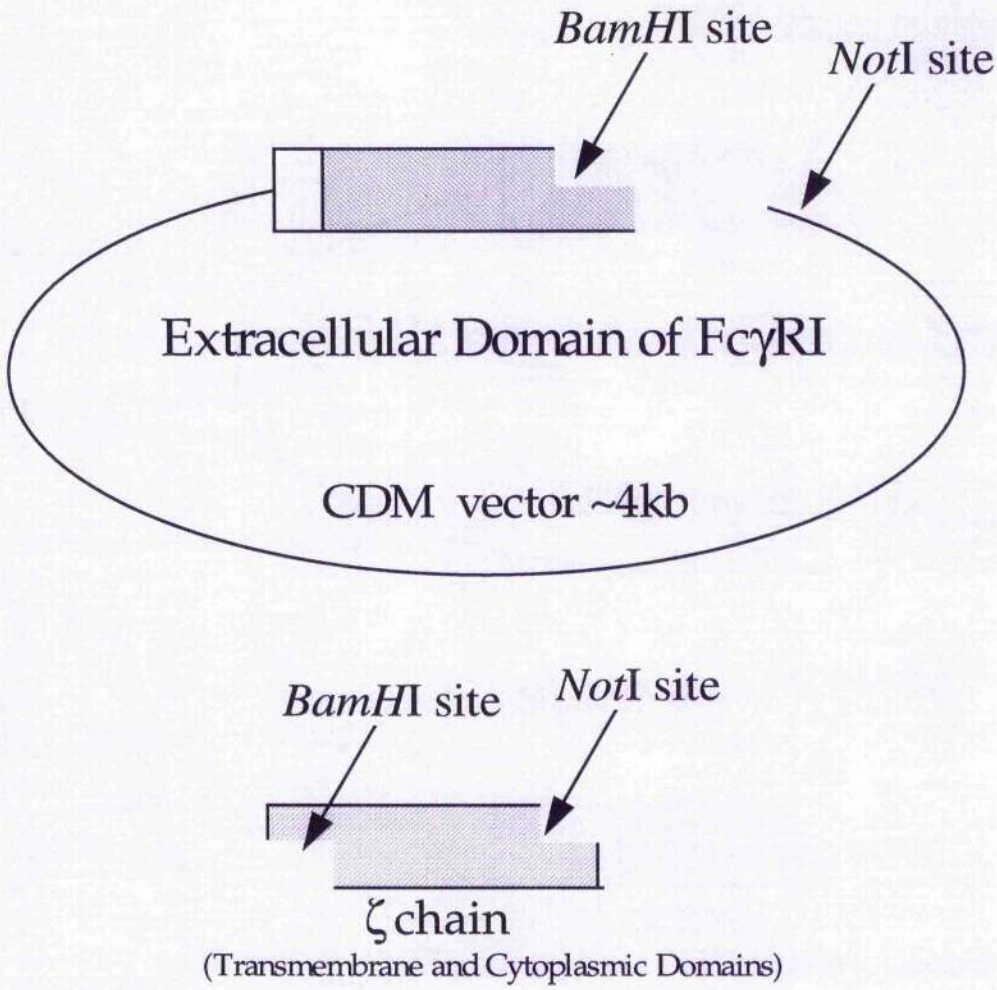
Figure 4.1 Construction of the FcγRI-ζ mutant receptor.

Step 1 The GPI segment of FcγRI-GPI in CDM is removed by doubly digesting the vector with *BamHI* and *Not*I restriction enzymes

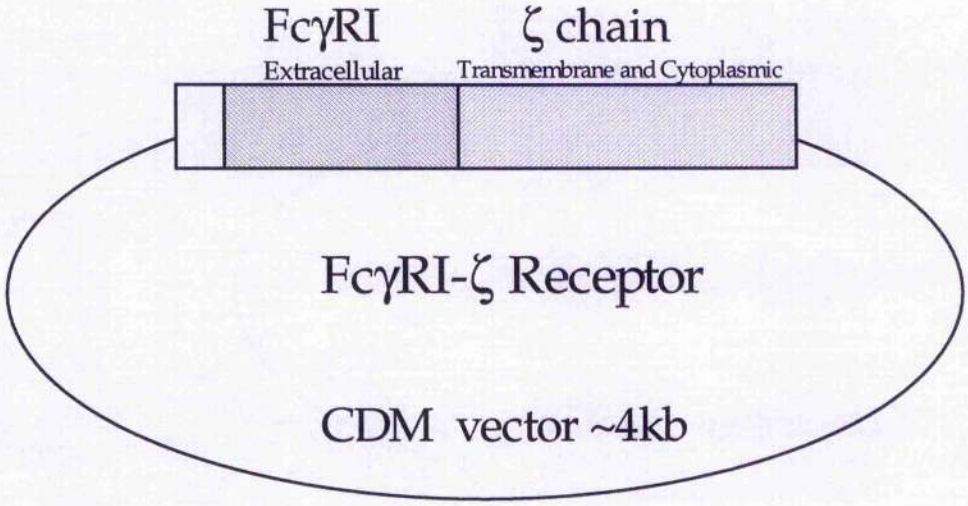


FP	Forward Primer
RP	Reverse Primer
GGATCC	internal *BamHI* site
GCGGCCGC	*NotI* site

Step 2 The zeta chain was amplified by PCR and simultaneously a *NotI* site was introduced at the 3′ end of the zeta chain so that it could be easily subcloned into the extracellular FcγRI containing CDM vector.



Step 3 The FcγRI extracellular and zeta chain transmembrane and cytoplasmic domains were ligated together in the CDM vector creating the FcγRI-ζ mutant receptor.



natural "in frame" *BamHI* site precisely at the junction between the extracellular domain and the transmembrane domain (bp 155 on the human zeta chain map, accession JO4132). This allowed for direct subcloning into the FcγRI-LFA3 CDM clone.

FcγRI-LFA3 (constructed previously by the Allen lab) was used to obtain the FcγRI extracellular region. In this construct a *BamHI* restriction site was created immediately following the extracellular region at the junction with the transmembrane domain. In the CDM vector a *NotI* restriction site immediately follows the insertion site for FcγRI-LFA3.

A double digestion of with FcγRI-LFA3 CDM and ζ-chain pTAG vectors with both *BamHI* and *NotI* for 1 hour at 37^0C was completed. The process separated the FcγRI-CDM fragment from the LFA3 fragment and the ζ-chain transmembrane and cytoplasmic domains from pTag-ζ chain extracellular respectively. The enzymes were heat deactivated (65^0C for 10 minutes). To facilitate subcloning the 5' phosphate groups were removed from FcγRI-CDM using shrimp alkaline phosphatase (standard protocol). The resultant fragments of FcγRI-CDM (of approximately 4.9 kb) and the ζ-chain transmembrane and cytoplasmic domains (of 447 bp) were electrophoresed and then excised from a 1% agarose gel. The DNA was retrieved using the chip dialysis method and elutip purified and then the ζ-chain fragment was subcloned into the FcγRI-CDM vector. Diagnostic cuts and then later sequencing experiments verified the identity of the novel FcγRI-ζ mutant (Figure 4.1).

4.8 Attempted Construction of the FcγRI/ζ Mutant With Only One in Three ITAM Motifs

Attempts were made to generate a FcγRI/ζ mutant with only the first of the 3 intrinsic ITAMs of FcγRI/ζ closest to the membrane, from now on referred to as FcγRI/ζ1. To do this there was an internal *Ava*II site in the ζ chain sequence following the first ITAM. A double digest was made using *NotI* to cut the plasmid after its ζ tail end, and the *Ava*II to cut the ζ2 and ζ3 intermittent sequence out from the plasmid before religation. The situation was complicated by an *Ava*II restriction site at position 1185 in the CDM vector.

A partial digest was performed to select a plasmid that had only been *Ava*II digested at the right location. Then 10μg of FcγRI/ζ CDM in 200μl was digested with 4 units of *NotI* (Boehringer Mannheim) in buffer B. Then 4

units of *Ava*II was added to the *Not*I linearized DNA while it was on ice, and then the sample was equally divided between 5 tubes. The tubes were heated to 37^0C for a time course of 0, 5, 10, 25, and 80 minutes. To each finished sample the magnesium chelating EDTA was added to a final concentration of 20mM. The desired band was cut from an agarose gel and purified by dialysis and elutiping (standard protocol). At this stage it was assumed that the ζ2 and ζ3 containing fragments were cut out, so a Klenow reaction was performed to convert the 5' overhangs into blunt ends. However, in this pilot experiment no sequencing reaction was performed at this time. The samples were ligated back into CDM with 1 unit of T4 DNA ligase and left overnight at 15^0C. The ligation reactions were then transformed into MC1061's and plated onto ampicillin and tetracyclin containing culture plates. The colonies retreived were miniprepped, but the desired product was never recovered, as assessed by diagnostic restriction enzyme cutting.

4.9 Validation of the Phagocytosis Assay

The FcγRI-ζ chain chimera was transiently transfected into COS-7 cells along with the previously constructed FcγRI-II and FcγRI-gamma chain (subunit of the FcεRI receptor) with various src and syk family kinases to examine their relative phagocytic ability.

Since COS-7 cells are not monocytes, there has been some debate as to whether phagocytosis assays conducted using this cell line may misrepresent normal monocyte receptor function. It has been suggested that the simian COS-7 cell cytoplasm may lack specific tyrosine kinases and specific machinery to correctly mimic Fc activation events on monocytes. Also, over-expression of Fc on the COS-7 cell surface yields up to 10,000 receptors per transfected cell versus the normal few present on monocyte cell surfaces.

Although these disadvantages must be taken into account, the COS-7 cell model for phagocytosis is now widely used. This is primarily because U937 monocyte-like cells have indigenous expression of FcγR's I and II, whereas COS-7 cells do not. Even if a U937 knock out line was developed, U937 cells are difficult cells to transfect with genetic material, unlike COS-7 cells.

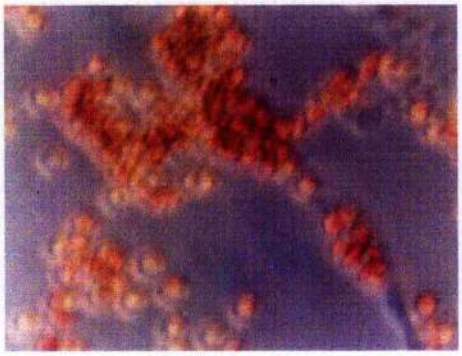
4.10 Mutant FcγRI/ζ Shows Rosetting and Phagocytosis Capabilities

Figure 4.2

A

B

C



D

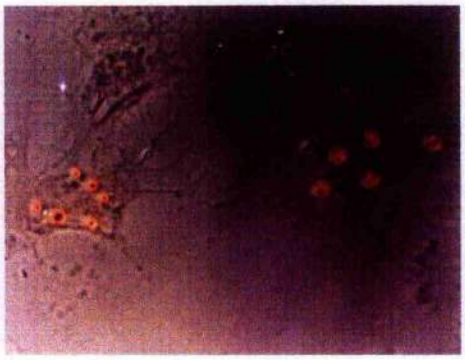


Figure 4.2 The FcγRI-ζ (Bryony's clone) was tested for its ability to phagocytose sheep red blood cells (SRBC) in a well established phagocytosis experiment using COS-7 cells (a simian kidney fibroblast cell line). **A** The control frame with no transfected Fcγ receptor. **B** FcγRI transfected cells. **C** Rosetting mediated by FcγRI-ζ mutant receptor. **D** Phagocytosis mediated by the FcγRI-ζ mutant receptor.

The FcγRI-ζ chain chimera was transiently transfected into COS-7 cells to see if the mutant was capable of performing the standard rosetting and phagocytosis assay. COS-7 cells transfected with any FcγRI extracellular cDNA leads to the surface expression of this Fc attachment specific region. IgG constant region specific Ab coated SRBCs will recognise and bind readily to this surface receptor. Phagocytosis will only occur substantially if the expressing receptor contains, or can recruit, an ITAM signalling domain that can recruit the necessary downstream molecules. Hyposhock procedures followed by an acid wash allows for visualisation of internalised cells after staining. As can be seen in Figure 4.2, FcγRI-ζ is shown to be capable of rosetting and phagocytosis, indicative of an intact and functioning signalling domain.

4.11 Validation of the Phagocytic Index

Arising from each assay there are three sets of functional and quantifiable data that can be calculated; average internalisation, percentage positive cells, and the phagocytic index (P.I.).

Average Internalisation shows the mean number of sheep red blood cells found inside the first twenty positive COS-7 cells. A positive cell is one that has internalised at least one SRBC. Within one transfection experiment average internalisation can be used to compare relative phagocytic ability. For each average internalisation result standard error was calculated from the formula; taking the square root of PxQ/n, which is based on the assumption of a binomial distribution.

Percent positive cells is the number of cells out of the first 100 cells that have internalised at least one SRBC. Since transfection rates differ, again it is best to compare relative percentage positive cells within one transfection experiment.

Although average internalisation and percent positive cells provides a valid measurement of phagocytosis within the same transfection experiment, for comparisons between transfections a new calculation was developed called the phagocytic index. The phagocytic index (PI) is the multiplication of the mean number of internalised SRBCs per positive COS-7 cell (± SEM) and the percent positive cells on a transfection plate (a positive cell having engulfed at least one SRBC) (Table 4.1). It shows the total number of SRBC found inside every one hundred COS-7 cells in a particular plate. This allows for quantitative comparisons of internalisation between transfections.

TABLE 4.1

Chimera Transfected	Average Internalisation	Percentage Positive Cells	Phagocytic Index
FcγRI	1.50 ± 0.61	2	3.0 ± 1.21
FcγRI + γ	5.30 ± 1.30	12	63.6 ± 15.62
FcγRI + ζ	9.60 ± 1.76	10	96.0 ± 17.59
FcγRI/γ	9.80 ± 3.21	17	166.6 ± 54.49
FcγRI/ζ	6.35 ± 1.60	11	69.9 ± 17.58
FcγRI/II	5.65 ± 0.99	12	67.8 ± 11.86

Standard Deviation for both average internalisation and the phagocytic index were calculated using Excel Version 4 (Microsoft).

4.12 Relative Phagocytosis of Selected Receptors and Signalling Molecules

After the ability of the FcγRI-ζ mutant receptor to mediate phagocytosis was investigated, comparative studies were done on phagocytic ability of several different mutant receptors, and wildtype receptors cotransfected with signalling subunits. Cell layers of COS-7 cells were transfected with FcγRI alone, FcγRI and γ-chain, FcγRI and ζ-chain, FcγRI/γ, and FcγRI/ζ as described in the materials and methods section. All FcγRI receptors were expressed and avidly bound opsonized SRBCs. This was confirmed by rosetting, as all wells of these experiments rosetted equivalently. However, the expression levels of cotransfected signalling subunits could not be assessed by rosetting. The results for 5 different experiments were pooled and the phagocytosis index was calculated and the results were graphed (Figure 4.3). Transfections of COS-7 cells with FcγRI and signalling subunits in all cases increased the phagocytic index from control levels.

4.12.1 The FcγRI + ζ Showed the Highest PI and Demonstrated a Better Phagocytic Ability Than Either FcγRI + γ or FcγRI/γ

This is interesting as ζ chain has 3 copies of the ITAM signalling motif compared with γ chain's one. Previous studies showed that chimeric

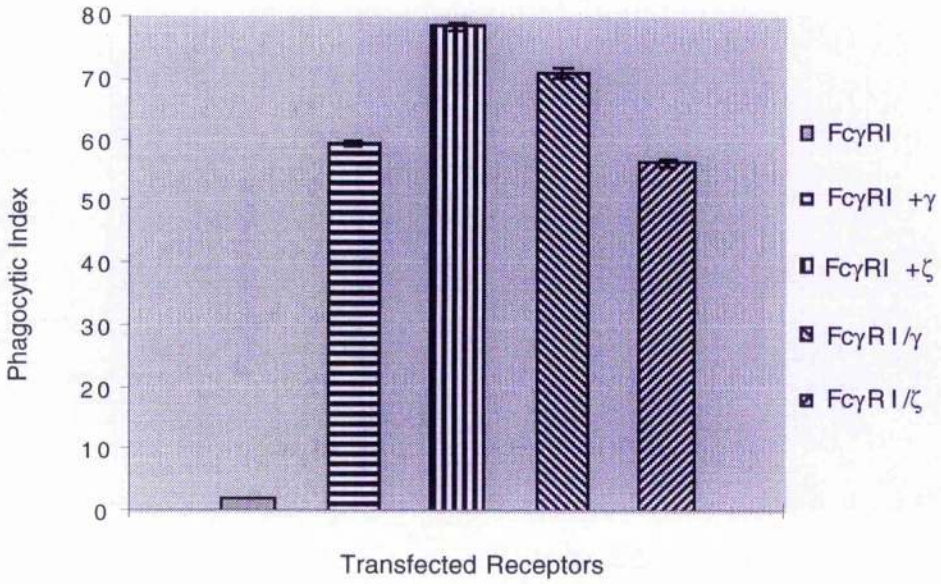


Figure 4.3 A comparison of the Phagocytic Index of FcγRI, FcγRI + γ, FcγRI + ζ, FcγRI/γ, and FcγRI/ζ. Each column is the cumulative average internalisation values over 5 separate experiments. The error bars are standard error.

receptors, each constructed to contain 1 of each of the 3 ITAM copies of the ζ chain functioned identically, but all at a lower activation level than the wild-type ζ chain **(Romeo *et al.*, 1992)**. Since each single ITAM ζ chimera acted equivalently to the ζ chain wild-type, this implicates ζ's multiple receptor ITAMs in signal amplification.

4.12.2 FcγRI/γ Was Significantly More Efficient at Phagocytosis Than FcγRI + γ

Previous work in the lab revealed that multiple cDNA's transfected into the same COS-7 cell decrease overall copy numbers of cDNAs expressed. Perhaps this can be explained by saturation of the cellular transcription machinery needed for plasmid expression. This may reveal why transfections with 10μg of the single FcγRI/γ plasmid were more effective than double transfections of both FcγRI and γ chain.

4.12.3 The FcγRI/ζ Mutant Was Found Less Capable of Phagocytosis Than FcγRI and ζ Transfected Separately

These results contradict previous findings that multiple cDNA's transfected into the same COS-7 cell decrease overall cDNA expression (as mentioned above). In an experiment conducted by Romeo et al., 1992, a zeta chain chimera containing only one out of the three ζ chain ITAMs showed the same activation events as the wild-type ζ chain, but were 8 fold less active than the full length ζ chain chimeras **(Romeo *et al.*, 1992)**. It was suggested that this reduced activity may be due to the chimera construction that disrupts di-sulphide linked dimer formation. Perhaps this would explain the observations in these COS-7 studies. Di-sulphide bond disruption or other elements critically absent from the new FcγRI/ζ mutant may inhibit receptor aggregation or binding of downstream signalling molecules. This would explain the reduced phagocytic ability of the mutant.

4.12.4 Control FcγRI Transfections in COS-7 Cells are Capable of Limited Phagocytosis

As can be seen, the occasional opsonized SRBC cell was internalized by the FcγRI transfected COS-7 cells. The FcγRI alone lacks an internal ITAM

signalling motif, and theoretically should be unable to phagocytose. This background phagocytosis in the control can be explained in a number of ways.

Perhaps the "positives" were a result of SRBCs shielded in some way from hypotonic shock conditions, leaving them attached to the perimeter of the COS-7 cells. Or, perhaps over the several hour incubation COS-7 cells enveloped SRBCs during normal growth and expansion.

FcγRI alone has been shown to capable of mediating immune complex endocytosis, a TK independent process. Perhaps at the moment of hypotonic shock several SRBCs were partially enveloped by the COS-7 cells in a recycling process, and were thus protected from hypotonic shock. Or lastly, by some unknown mechanism, FcγRI is actually able to mediate some low level phagocytosis.

4.12.5 Summary of Individual Receptor Phagocytic Ability

Cotransfection experiments with FcγRI and either γ or ζ chain showed a dramatic increase in the number of COS-7 cells able to phagocytose SRBCs. The mean phagocytic index was also dramatically increased. This is interesting as it shows cDNAs for both γ and ζ chain are expressed to a great enough extent in COS-7 to support FcγRI mediated phagocytosis. This is important because rosetting does not verify expression of cytoplasmically located proteins.

It is also clear that the endogenous COS-7 tyrosine kinases are able to interact with the ITAMs on these accessory molecules and initiate downstream signalling events. This demonstrates a certain degree of conservation of this signalling process. In any case, it is interesting that in this simian fibroblast cell line, transfection with Fc receptors can cause it to exhibit macrophage-like behaviour.

4.13 Cotransfection Experiments Using FcγRI/γ, FcγRI/ζ and FcγRI/II Mutants

The cytoplasmic tail of FcγRII contains the prerequisite signalling components to initiate phagocytosis. FcγRI can associate with FcγRII transmembrane and cytoplasmic domains to initiate signalling. Previous experiments suggest that ligand binding acts to tether the target particle to the membrane surface and aggregate signalling domains in active cells. However, in this phagocytosis

experiment it was unknown whether the different extracellular domains of the high affinity FcγRI and low affinity FcγRII may import differences in phagocytic ability. This was undesirable as comparisons between the different cytoplasmic signalling components was being investigated. To eliminate such a question of variability, a chimeric receptor FcγRI/II was constructed in the lab (George's clone). It comprised the extracellular domains of FcγRI and transmembrane and cytoplasmic domains of FcγRII. This receptor was compared with the similarly constructed FcγRI/γ (Andre's clone) and FcγR/ζ (Bryony's clone) chimeras, so only signalling domains were being compared.

4.14 Cotransfection Experiments Testing the Effect of Lyn and Fyn Tyrosine Kinase Overexpression on FcγRI/γ, FcγRI/ζ, and FcγRI/II Mutants

As mentioned in the introduction, ITAM initiation stimulates a cascade of tyrosine kinases that mediate downstream effector function. Several COS-7 transfection experiments were conducted to investigate whether distinct TK phosphorylation patterns were observed when receptor signalling was confined to using a specific ITAM. It is thought that different src family kinases initiate signalling cascades after binding distinct ITAMs (Figure 4.4). In each transfection experiment 10μg of each plasmid was added to a 50-70% confluent COS-7 well. Each well was done in duplicate.

4.15 The Effect of Tyrosine Kinases Lyn and Fyn on FcγRI/γ, FcγRI/ζ, and FcγRI/II Internalisation (Figure 4.4)

FcγRI/γ showed increased average internalisation when cotransfected with mLyn and slightly decreased average internalisation when transfected with hFyn. In contrast FcγRI/ζ mutant showed increased average internalisation when cotransfected with hFyn and decreased internalisation when cotransfected with mLyn. Lastly, cotransfections of both mLyn and hFyn with FcγRI/II decreased average internalisation in both cases. The increases and decreases from controls were within a small range of approximately ±2 internalised SRBCs for all mutants, which is not dramatic. Several observations can be discussed here.

It was interesting that cotransfections with mLyn increased FcγRI/γ internalisation rates. In B cells it has been shown that receptor clustering

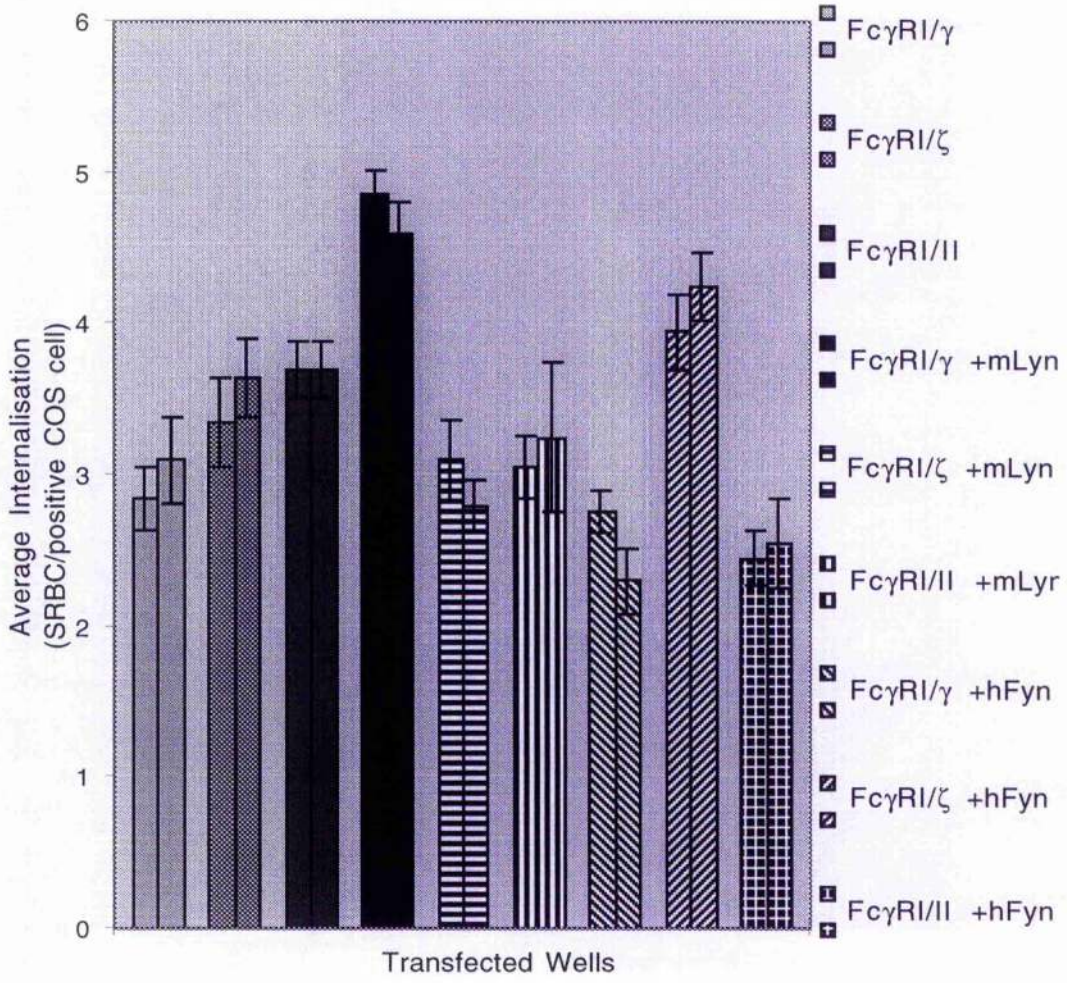


Figure 4.4 10μg of each receptor and associated subunit plasmids were added to a 50-70% confluent COS-7 plate. Either src-family kinase hFyn or mLyn was added in equal concentration, and average internalisation rates of these cotransfected wells were compared with controls. The experiment was repeated and the duplicate lane indicates the second set of results. The error bars are standard error.

following activation allows Lyn to phosphorylate and γ chain of FcεRI **(Amouri et al., 1997)**. Why hFyn cotransfections actually decreased rates of internalisation is harder to discern. Perhaps binding sites for hFyn do not exist on gamma chain, so the only effect of Fyn cotransfections was to clog transcription machinery in place of FcγRI/γ.

It is likewise interesting that cotransfections with FcγRI/ζ with hFyn increased internalisation rates slightly. The Fyn tyrosine kinase has been shown to have direct associations with the ζ chain prior to activation via its N-terminal 10 amino acids **(Gauen et al., 1992)**. In contrast, mLyn decreased internalisation rates, perhaps again because it did not bind the ζ chain and acted only to compete with the mutant receptor for COS-7 cell transcription machinery.

Lastly, both hFyn and mLyn cotransfections with FcγRI/II both decreased internalisation rates compared with controls. Evidence suggests that when FcγRII is stimulated on platelets, its ITAM tyrosines are rapidly phosphorylated the Src tyrosine kinase protein being strongly implicated **(Huang *et al.*, 1992)**. Perhaps cotransfection with the Src tyrosine kinase would be more effective at increases the average internalisation of FcγRI/II.

4.16 Increase of Src-family Tyrosine Kinase Relative to Receptor cDNA Transfected (Figure 4.5)

The amount of tyrosine kinases that are cotransfected in cells expressing threshold levels of receptors is hard to determine (as rosetting is not an indication). It was thought that perhaps cotransfecting large amounts of TK relative to receptor would ensure those cells capable of phagocytosis were likely to be coexpressing either mLyn or hFyn. In Figure 4.5, 20μg either mLyn or hFyn were cotransfected with 5μg of receptors and subunits.

4.17 3 cDNAs Transfected Significantly Reduced Average Internalisation Measurements (Figure 4.5)

When 3 types of cDNAs were cotransfected, there was significant reduction in average internalisation rates. Perhaps when receptors are transfected with FcγRI, signalling subunits, and tyrosine kinases, the transcription machinery of the cell may be so overloaded that one or more of the critical components of increased phagocytosis is not expressed at sufficient levels. It must be noted

that 30μg of 3 different types of cDNA is quite large for transfection experiments.

Perhaps the cDNA so overpowered the machinery of the COS-7 cells that they were hampered from expressing critical proteins necessary for their own survival, therefore they demonstrated failed phagocytic ability as well.

4.18 The Effect of Tyrosine Kinases mLyn and hFyn on FcγRI/γ, FcγRI/ζ, and FcγRI/II Internalisation (Figure 4.5)

The most interesting thing to note in these experiments was that large amounts of mLyn increased average internalisation for all three types of mutants, and having the largest effect on FcγRI/ζ. This contradicts the last set of results were mLyn was slightly better at aiding FcγRI/γ phagocytosis, in line with the current literature on the subject.

Lyn has been implicated in γ chain signalling of the FcεRI receptor. Perhaps the FcγRI cytoplasmic tail, although not containing any recognised signalling motifs, may contain other, as of yet unrecognised motifs that bind Lyn SH4 regions prior to activation. In this way Lyn is in close proximity of the γ chain ITAMs before cellular activation. Upon receptor aggregation Lyn binds via its SH2 domain to phosphorylated ITAMs of the accessory signalling proteins. This would explain why in the mutant FcγRI/γ chain Lyn may not confer the same advantage to the γ chain over ζ and II signalling domains in the absence of the FcγRI tail.

In contrast to Lyn, overloading hFyn transfection did not dramatically increase the average internalisation of any of the three mutants over one another. They all demonstrate relatively equivalent internalisation capabilities. There is an endogenous Fyn-like kinase expression reported in COS-1 cells (**Hall *et al.*, 1993**). Perhaps excessive cDNA expression of this tyrosine kinases alters expression of endogenous COS-7 cell proteins that affect phagocytosis. Perhaps there is some form of negative feedback loop whereby endogenous tyrosine kinase expression of Fyn is decreased in the presence of transfected Fyn, so the total contribution of Fyn kinases does little to effect levels of phagocytosis from controls.

4.19 The Effect of Tyrosine Kinase mSyk on FcγRI/γ, FcγRI/ζ, and FcγRI/II Internalisation (Figure 4.6)

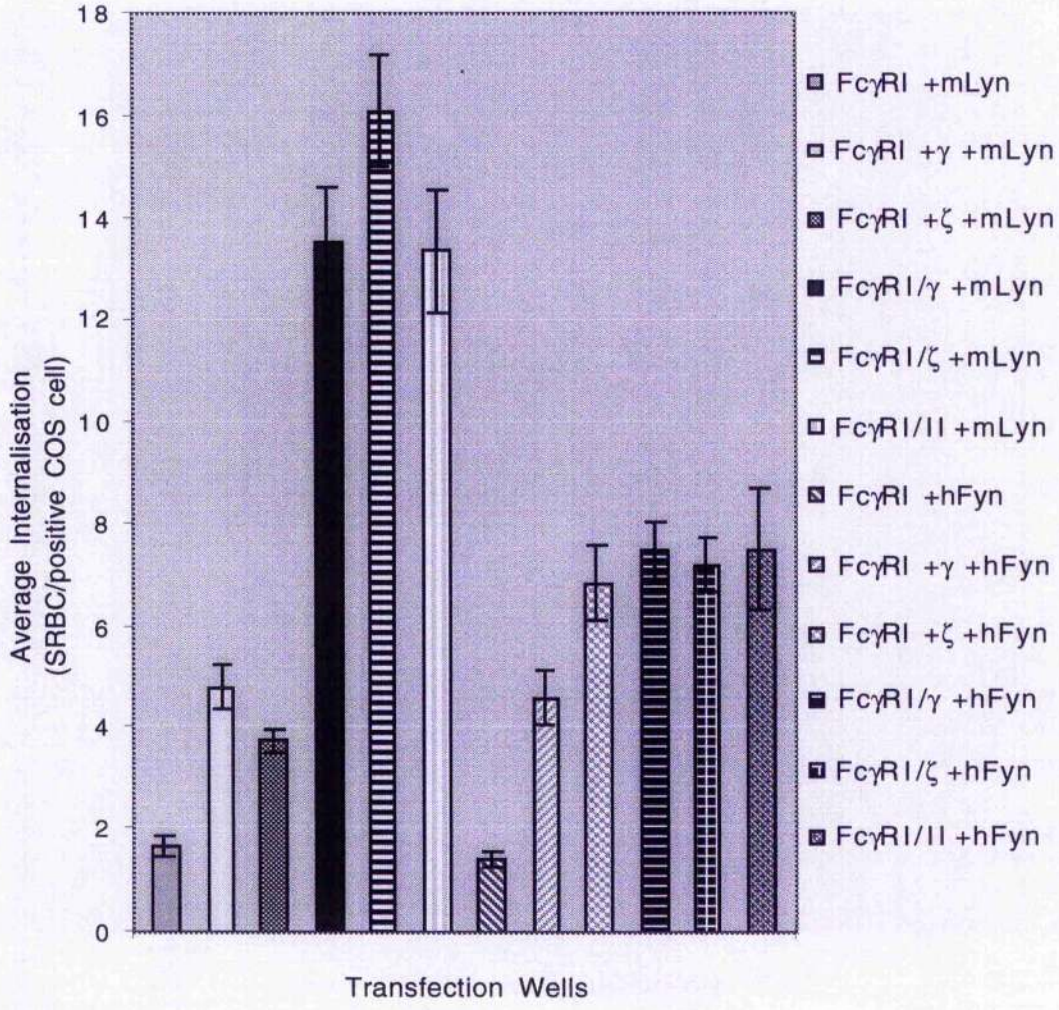


Figure 4.5 5μg of each receptor and associated subunit plasmids and 20μg of src-family kinases hFyn or mLyn were added to selected wells on a 50-70% confluent COS-7 plate. Average internalisation rates of these cotransfected wells were then compared with controls.

In many studies the important role of syk tyrosine kinases over src family tyrosine kinases has been established **(Kolanus *et al.*, 1993)(Greenberg *et al.*, 1996)**. Because of this it was decided to test the ability of mSyk in cotransfection experiments with mutant receptor FcγRI/γ, FcγRI/ζ, and FcγRI/II. In the first experiment 10μg of each receptor type (some signalling subunits) and Syk tyrosine kinase was cotransfected into COS-7 cells. Dramatic increases in internalisation and the percent positive cells was observed for all mutant receptors compared to controls. Indeed, in some cells internalisation was so efficient that the magnified appearance of the cells resembled rosetting. All the mutant receptors had comparable levels of phagocytosis. All the mutants, and especially the FcγRI/ζ mutant, demonstrated a high standard error measurement, as many transfection wells had some cells with one internalised SRBC and neighbouring cells containing 30^+ SRBCs. Perhaps the reason for this is that cells with a low number of internalised cells had only receptor and signalling subunits being expressed, while cells "bursting" with SRBCs were expressing Syk as well as receptor and signalling subunits. The next experiment was designed so that an increase of mSyk tyrosine kinase relative to receptor cDNA was transfected.

4.20 Increase of mSyk Tyrosine Kinase Relative to Receptor cDNA Transfected (Figures 4.7, 4.8, and 4.9 (PI))

In this experiment 2.5μg of receptor and signalling subunits, and 20μg of mSyK was transfected into COS-7 cells (Figures 4.7 and 4.8). Again Syk cotransfected cells show some cells having phagocytosed to such an extent that they are bursting with SRBCs. The degree to which this is observed is phenomenal. In particular Figure 4.8 best shows the dramatic increase in internalisation compared with all controls. The percent positive cells is also dramatically increased in all cotransfection with signalling domains and Syk.

However, there was still a wide range of internalisation values, although less cells with just one internalised SRBC were observed compared with the last experiment. Interestingly, when counting the percent positive cells that have internalized 10 or more SRBC, the numbers are close to the percent positive cells that have internalised at least one SRBCs during normal Syk-transfections (Table 4.2). This suggests Syk somehow decreases the threshold of internalisation, so cells that normally internalise small numbers of SRBCs begin to internalise large numbers, and many cells that previously internalised no SRBCs at all are now capable of some internalisation.

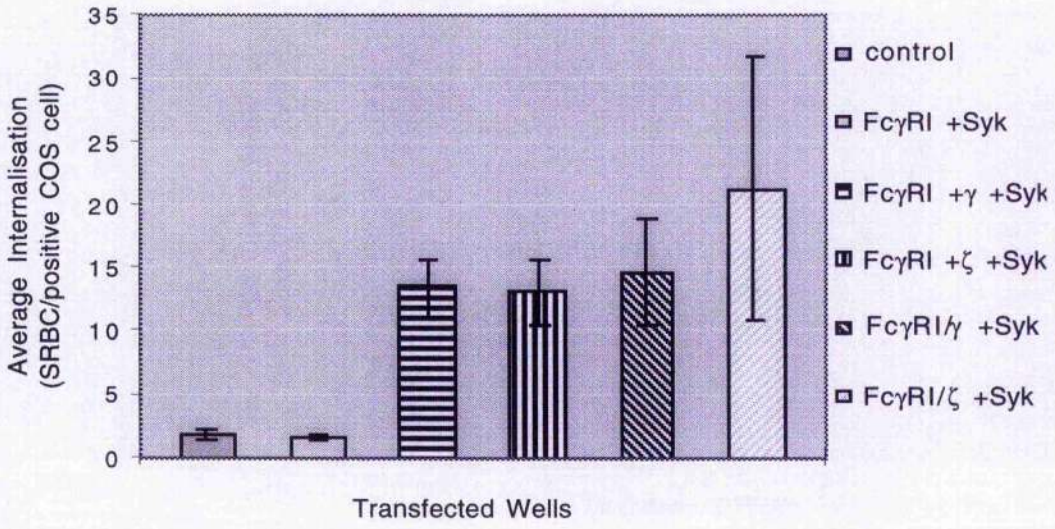


Figure 4.6 10μg of each plasmid and associated subunit plasmids were added to a 50-70% confluent COS-7 plate. The mSyk plasmid was added in equal concentration to all wells apart from the control. Average internalisation rates of these cotransfected wells were then compared.

Figure 4.7

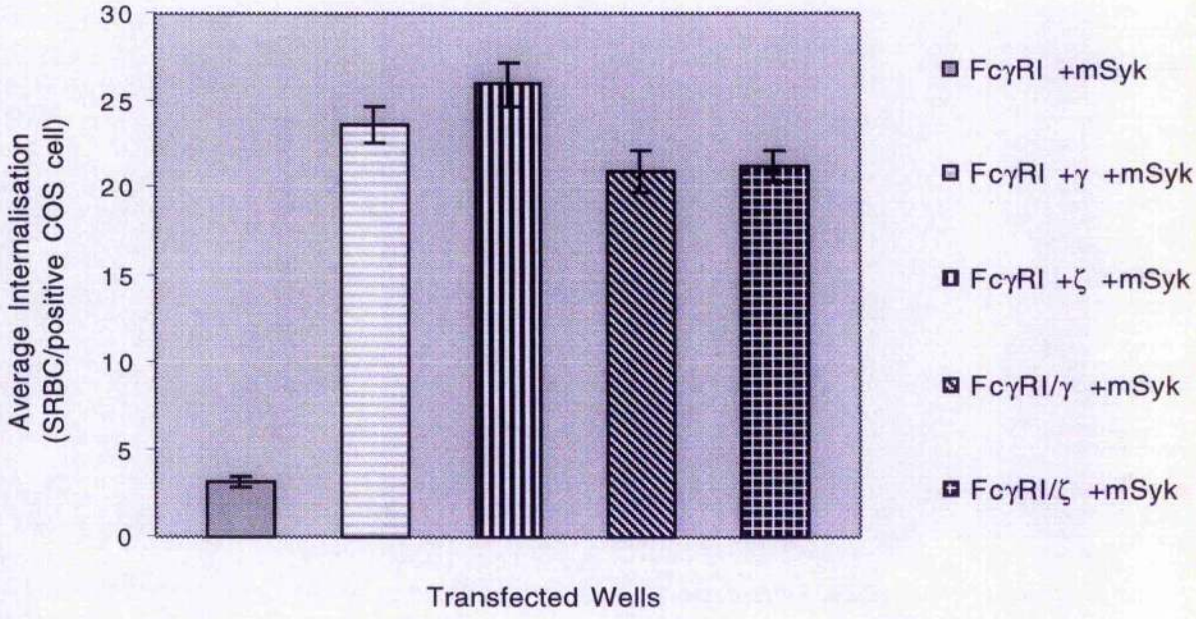


Figure 4.8

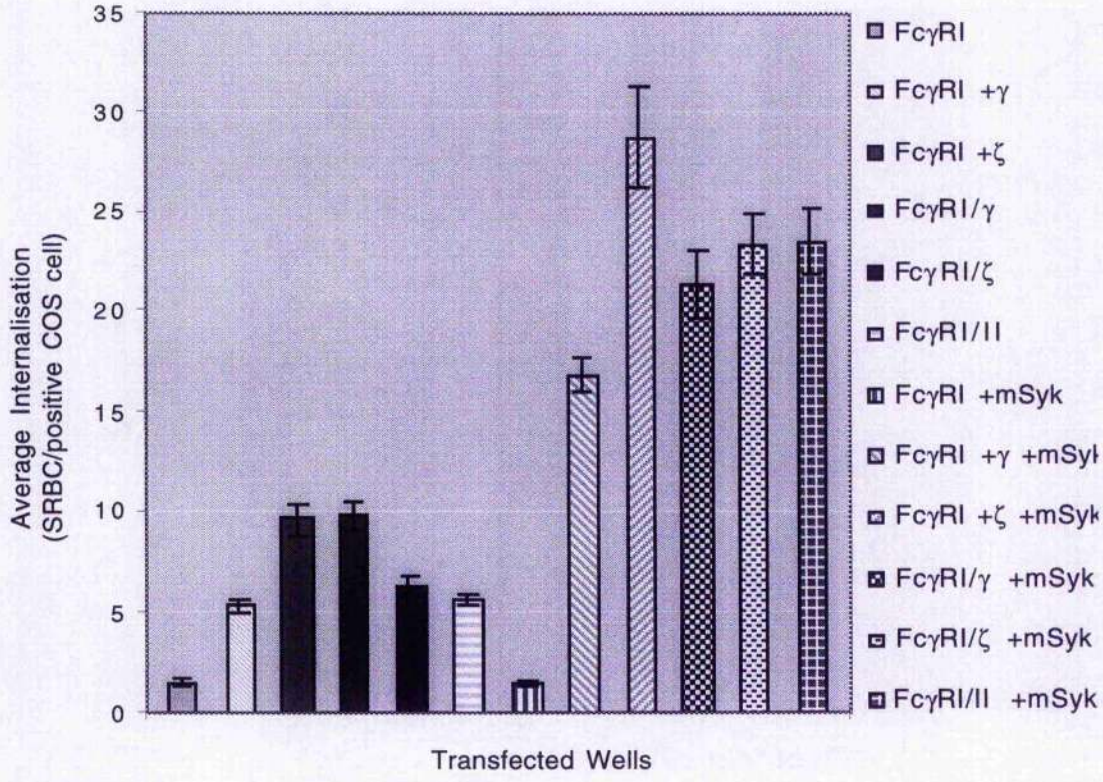


Figure 4.7 2.5μg of each receptor and associated subunit plasmids and 20μg of the mSyk plasmid were added to all wells on a 50-70% confluent COS-7 plate. Average internalisation rates of these cotransfected wells were then compared.

Figure 4.8 2.5μg of each receptor and associated subunit plasmids and 20μg of the mSyk plasmid were added to selected wells on a 50-70% confluent COS-7 plate. Average internalisation rates of these cotransfected wells were then compared with controls.

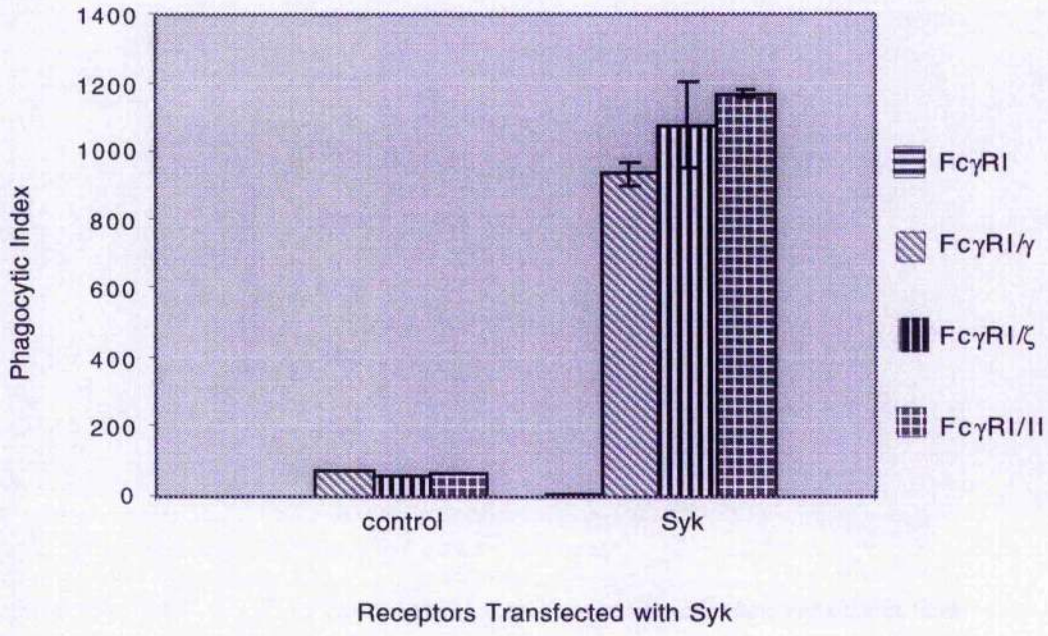


Figure 4.9 The Phagocytic Index (PI) was calculated for mutant receptors cotransfected with mSyk in all the experiments involving mSyk mentioned thus far. These PI's were then compared with each other and with the control.

Table 4.2

Chimera and TK Transfected	Percentage Positive Cells	Percentage Positive Cells (>10 each)
FcγRI/γ	13	0
FcγRI/ζ	14	0
FcγRI/II	16	0
FcγRI/γ + Syk	33	15
FcγRI/ζ + Syk	43	11
FcγRI/II + Syk	38	11

The Phagocytic Index was calculated for all experiments involving cotransfections with mSyk (Figure 4.9). For all three mutants FcγRI/γ, FcγRI/ζ, and FcγRI/II PI was dramatically increased with mSyk involvement relative to controls. FcγRI/II had the largest PI, while FcγRI/ζ was second as effective, and lastly FcγRI/γ. The abilities of all to phagocytose were at comparable levels relative to controls.

4.21 A Final Experiment Cotransfecting with All Types of Tyrosine Kinases (Figure 4.10)

In this final experiment 5μg of mutant receptor was transfected along with 10μg of tyrosine kinase cDNA. The experimental wells were done in duplicate. As expected the mSyk tyrosine kinase was the most effective at increasing internalisation for all three mutants, and at approximately equivalent levels. The mLyn also increased phagocytosis slightly compared to controls for all three receptors. The hFyn tyrosine kinase was the least effective at increasing phagocytic ability between all three receptor types.

Some experiments conducted showed differences in the tyrosine kinases recruited by distinct receptor types. However, it is more easily observed that particulartyrosine kinases are better than others at aiding internalisation of ITAM-mediated phagocytosis in COS-7 cells. Specifically mSyk significantly increased PI for all mutnat receptor types tested. For the mutant FcγRI/ζ photgraphs of cells were taken to shows its ability to phagocytose when co-transfected with the various tyrosine kinases (Figure 4.11).

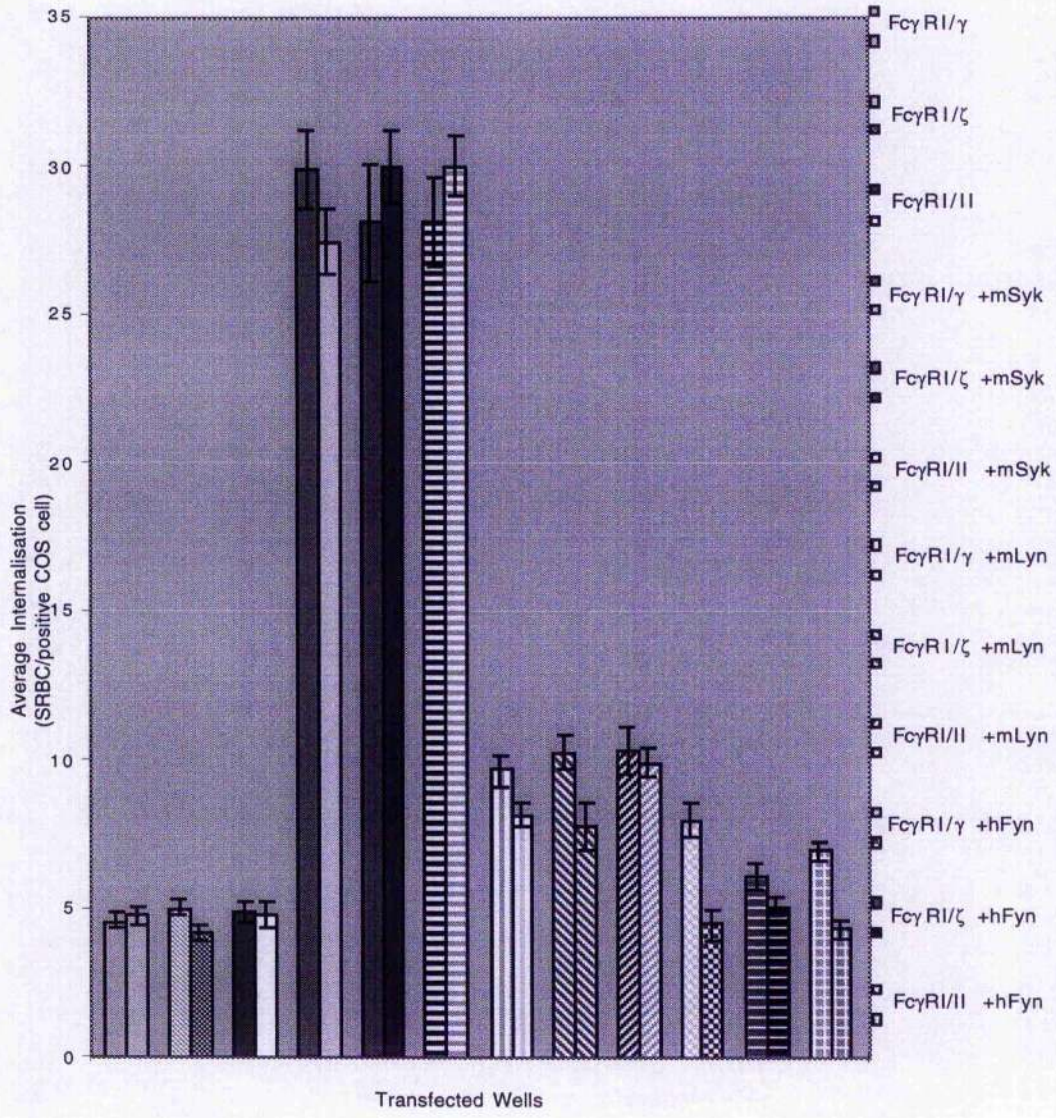


Figure 4.10 5μg of each mutant receptor plasmid and 10μg of specific tyrosine kinase cDNAs were cotransfected into 50-70% confluent COS-7 cell plates. The experiment was done in duplicate. Resulting average internalisation rates were compared between receptor types.

Figure 4.11

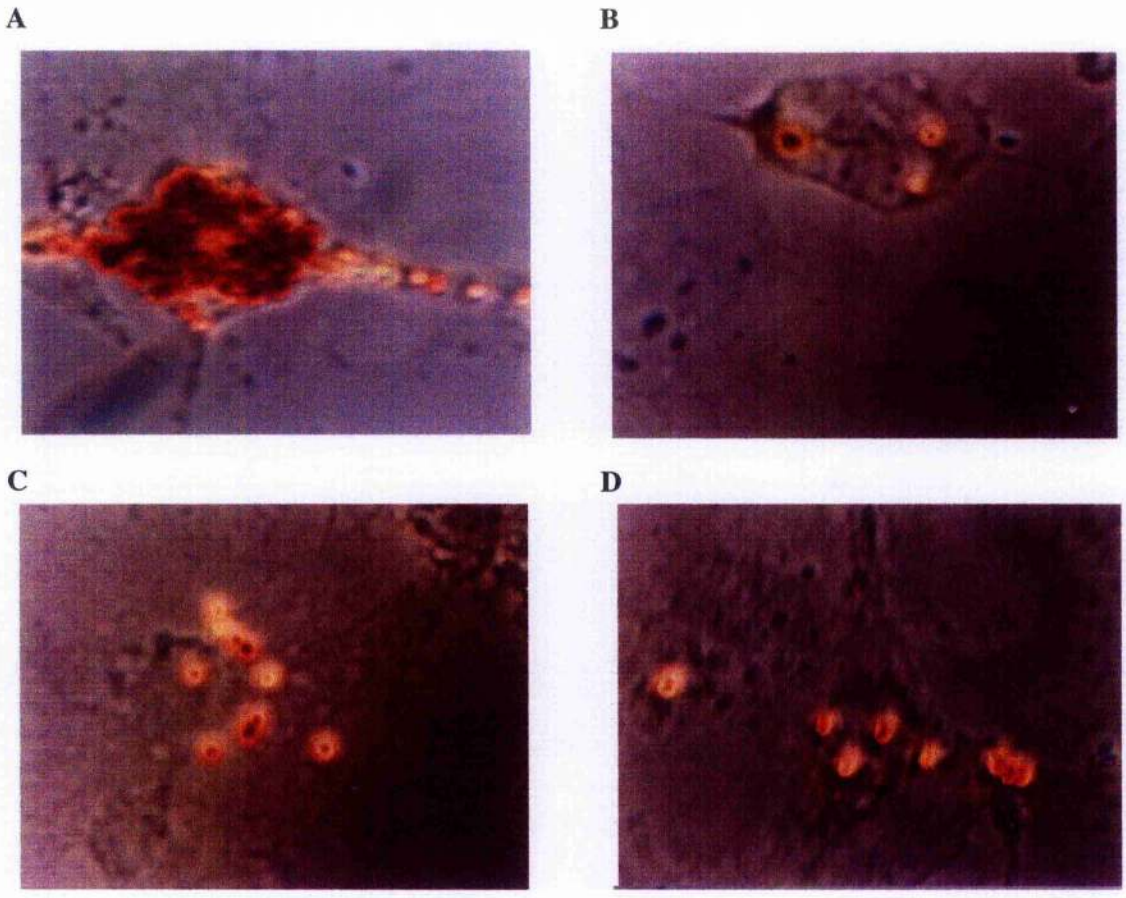


Figure 4.11 The FcγRI-ζ (Bryony's clone) was cotransfected into COS-7 cells with tyrosine kinases mLyn, hFyn, and mSyk. The relative ability of FcγRI-ζ to phagocytose sheep red blood cells (SRBC) in the presence of these tyrosine kinases was investigated. **D** Phagocytosis mediated by FcγRI-ζ alone. **C** Phagocytosis mediated by FcγRI-ζ cotransfected with mLyn. **B** Phagocytosis mediated by FcγRI-ζ cotransfected with hFyn. **A** Phagocytosis mediated by FcγRI-ζ cotransfected with mSyk.

Chapter 5

Discussion

5.1 Western Discussion Section

5.1.1 Samples Probed with Anti-Syk Antibody

There are several reasons why it was decided to probe with anti-Syk antibody to test for differential recruitment of this tyrosine kinase at the early stages. The zeta chain of the TCR and the gamma chain of FcεRI in B cells and FcγRI in monocytes were all found able to recruit Syk family kinases post activation **(Johnson et al., 1995)**. Syk-deficient macrophage cells are unable to phagocytose, demonstrating the importance of this tyrosine kinase in particular **(Takata *et al.*, 1994)(Greenberg *et al.*, 1995)**. Also, chimeric receptors with exclusively Syk cytoplasmic domains were able to induce cytoskeletal rearrangements and full phagocytic activity in COS-7 cells. The tyrosine kinase Syk was in this way implicated in having an extremely important role in cell signalling **(Greenberg *et al.*, 1996)**.

In both the FcγRI and FcγRII signalling lysates, Syk at 72kDa was a prominent tyrosine kinase found in non-resting cells. Seemingly, although initial receptor engagement may result in alternative pathways via distinct ITAMS, ultimately the FcγRI and II pathways overlap, with the consequent Syk phosphorylation resulting. Interestingly, it is the ZAP-70 syk-like protein kinase in T cells and NK cells which may have the same function in hematopoietic cells of this range.

5.1.2 Differential Syk Recruitment in IFNγ and DBC Treated Cells

In these preliminary experiments probing for Syk tyrosine kinase recruitment, a differential pattern was observed for IFNγ and DBC treated cells. As mentioned before, IFNγ treated cells have FcγRI and γ chain upregulation, while DBC treated cells have FcγRII upregulation. While IFNγ cells show peak Syk activation 5-7 minutes post activation that is still high 30 minutes later, DBC cells show an immediate increase in Syk recruitment following activation that is tailing off by 30 minutes post-activation. This suggests

that the FcγRI and γ-chain signalling mechanism has a delayed activation of Syk compared with FcγRII's mechanism.

Interestingly, in HL60 cells engagement of FcγRI induced tyrosine kinase phosphorylation that peaked at 5-10 minutes after activation and returning to basal levels by 60 minutes. In contrast, engagement via the FcγRII receptor induced rapid tyrosine phosphorylation that declined to basal levels by 40 minutes **(Agarwal *et al.*, 1993)**. This previous result helps support these latest findings.

5.1.3 Tyrosine Kinases Directly Linked to Ca^{2+} Release Patterns

Other studies have critically linked tyrosine kinase recruitment with increases in Ca^{2+} release patterns post-activation. Rapid phosphorylated TK augmentation was detected in THP-1 cells thirty seconds after activation, followed by Ca^{2+} increases observed soon after at 1-2 minutes **(Rankin *et al.*, 1993)**. Herbimycin-A, Genistein, and Erbstatin all block TK activity and when added to activated cell cultures of THP-1 cells, both TK phosphorylation and intracellular free Ca^{2+} were reduced **(Rankin *et al.*, 1993)**. This suggests that the pathway to Ca^{2+} release stimulated by FcγR's may be mediated through cellular mechanisms involving tyrosine kinases.

In Syk deficient B cells PLC-γ2 failed to become phosphorylated and the IP3 turnover and calcium mobilisation was severely hampered **(Takata *et al.*, 1994)**. In Lyn deficient B cells, while Syk phosphorylation was profoundly reduced **(Kurosaki *et al.*, 1994)**, IP3 turnover was identical to that of wild-type **(Takata *et al.*, 1994)**. However, Ca^{2+} mobilization was delayed, implying a second IP3-independent mechanism for Ca^{2+} mobilization **(Takata *et al.*, 1994)**. Either that or other B cell src proteins were capable of substituting for function, namely Fyn, Blk, or Lck.

5.1.4 Ca^{2+} Release Patterns Compared With Syk Recruitment

Interestingly, the differential Syk recruitment in IFNγ and DBC treated cells corresponds to differences in Ca^{2+} release patterns in the two cell types previously observed in the laboratory of Professor J. Allen. After receptor cross-linking, IFNγ and DBC treated cells do not exhibit a common pathway of store-mediated Ca^{2+} entry. Magnitude, kinetics, duration and relative contribution of internal and external Ca^{2+} stores are different between the differentiated U937 cells **(Davis *et al.*, 1994)**.

The increase in calcium ions after cellular activation is not exclusively supplied by increase of internal stores release. The response is also partly due to influx of external calcium. Ca^{2+} cytosolic increase after cross-linking was observed in IFNγ U937 cells. A more prolonged increase from baseline cytosolic Ca^{2+} was observed in dibutyl cAMP treated cells. While in both cases Ca^{2+} elevation results from both external and internal sources, the peak IFNγ response is mainly due to intracellular release, while the DBC response is attributed to external influx of Ca^{2+} **(Davis *et al.*, 1995)**. It is speculated that FcγRII may act itself or regulate directly plasma membrane Ca^{2+} channels. Other surface receptors have been shown capable of external ion channel regulation. For example, multimeric complexes of CD20 has been shown to be a novel receptor directly regulating Ca^{2+} ion channel conductance in B cells. Stable transfection of CD20 cDNA into both human T and pre-B lymphoblastoid cell lines substantially increased transmembrane Ca^{2+} conductance **(Bubien *et al.*, 1993)**.

Monitoring Ca^{2+} responses to FcγR cross-linking in differentiated U937 cells has shown different responses of the FcγRI high affinity and FcγRII low affinity receptor. This evidence implicates the two receptor types in initiating unique Ca^{2+} release mechanisms. This is perhaps by the recruitment of distinct TKs to the different ITAMs responsible for intracellular signalling. Different Syk TK post activation patterns have now been seen for the two receptor types. What the mechanism is by which Syk recruitment is immediate in DBC treated cells and delayed in IFNγ cells has not been determined. Perhaps the events are regulated by upstream src-tyrosine kinase specific signalling. The alternative pathway for post Syk events leading to different Ca^{2+} release patterns also awaits elucidation.

5.1.5 Alternative Pathway for Differential Ca^{2+} Release

Interestingly, it was recently discovered an alternative signalling pathway in the rat mast cell line RBL-2H3 after FcεRI aggregation on the cell surface **(Kinet, 1996)**. In this pathway, activated receptors in turn activate sphingosine kinase. This kinase activates sphingosine-1-phosphate, a potent secondary messenger that signals for Ca^{2+} release from intracellular stores **(Beaven, 1996)**. Indeed Kinet found this to be the major contributing pathway of Ca^{2+} stores release in this cell line **(Kinet, 1996)**. The alternative and widely recognised Syk activating mechanism via IP3 was a minor contributor in comparison. The SK pathway may lie downstream of tyrosine kinase

activation, however, this has yet to be proven. It has yet to be determined to what location of the FcεRI the sphingosine kinase binds, however, if its attachment is to the γ chain or ITAM in general, this observation may have important implications in FcγR signalling, and may help to explain observed differences in Ca^{2+} responses following FcγRII versus FcγRI triggering.

5.1.6 Are Src Family Proteins the Ones Found Phosphorylated in DBC and IFNγ Lysates

In the experiment where blots of lysates of INFγ and DBC cells were probed with anti-phosphotyrosine antibody (Figures 3.1.6 and 3.1.7), the identity of the 110kDa bands on IFNγ and DBC blots, and 50kDa band, found exclusively on the DBC blot remains unknown. Reprobing with a general anti-src did not help elucidate the bands identity. Interestingly, other studies have found the predominant phosphorylated protein in FcγR activated lysates to be of 115kDa **(Agarwal *et al.*, 1993)**. Following cross-linking of murine IgC2a or human IgG to FcγR on monocytes THP-1 line, TK phosphorylation was present including a doublet of 110kDa and bands of 80, 60, and 52kDa and other proteins that increased their rapidity and degree of phosphorylation depending the dose of cross-linking antibody added **(Rankin *et al.*, 1993)**. Future experiments could try to discern the identity of the 50kDa band present in DBC treated cells and not in IFNγ treated cells.

In general it has been determined that the distinct TK recruitment pattern of different ITAMs in select cell lines may have a clear role in activating distinct effector functions. So far the recent work focused on Syk activation and a differential pattern of recruitment for FcγRI γ-chain and FcγRII expressing cells has been found. However, the src-family TKs that may be involved in binding distinct or overlapping sets of substrates awaits elucidation. It is now very much doubted that distinct kinases serve redundant functions.

5.2 Nitric Oxide Discussion Section

There are several reasons that could explain why the Griess reaction did not detect nitric oxide production. Perhaps the incubation time of 3 hours with opsonized SRBCs was not long enough to accumulate substantial amounts of nitric oxide following phagocytosis. Also perhaps the heme protein from the SRBCs used to activate macrophage FcγRs interfered with detection of nitric

oxide. It was thought that perhaps using a large FcR specific antigen this could activate macrophage cells and stimulate them to produce nitric oxide without the repercussions of the heme protein.

The main reason why hNosi proteins were not detected by western probing of DBC and IFNγ cell blots may have been because they were not in a high enough state of activation prior to cell lysis, so that levels of hNOSi proteins were not high enough to be detected. Another reason why western probing may have been unsuccessful was that perhaps the primary antibody was too dilute to get an appreciable signal after just an hour incubation. Perhaps the polyclonal itself, a generous gift from Professor E. Liew's lab, was old and degraded with use. Also, perhaps another type of lysis buffer beside the weak RIPA buffer would have been better able to dissociate cellular components in this experiment. Further experiments to detect differences in nitric oxide production between DBC and IFNγ U937 can be done in the future. One might include RT PCR on lysed activated monocytes to search for hNOSi messenger RNA. In all future experiments it would be useful to find a suitable positive control for hNOSi proteins.

5.3 Phagocytosis Discussion Section

5.3.1 Relative Receptor Phagocytic Ability

The novel FcγRI-ζ was capable of rosetting and phagocytosis, indicative of an intact and functioning signalling domain. Transfections of COS-7 cells with FcγRI and signalling subunits in all cases increased the phagocytic index from control levels.

The FcγRI + ζ demonstrated a better phagocytic ability than either FcγRI + γ or FcγRI/γ. While FcγRI/γ was significantly more efficient at phagocytosis than FcγRI + γ. The FcγRI/ζ mutant, while exhibiting a dramatic increase in PI compared to controls, was found less capable at phagocytosis than when FcγRI and zeta were transfected separately.

5.3.2 FcγRI Control Showed Background Phagocytosis

Control FcγRI transfections in COS-7 cells are capable of limited phagocytosis. Although it may be do to low level phagocytosis, or several of the other reasons mentioned in the results, it would be interesting to test if this background internalisation is yet another example of FcγRI endocytosis.

Such endocytosis has been shown to take place in the absence of transmembrane and cytoplasmic domains, but seemingly depends on extracellular components of the receptor **(Harrison *et al.*, 1994)**. The molecules present in both the U937 and COS-7 cell lines involved in this process remain unknown, although clathrin associating adaptor proteins have been implicated.

Clathrin which is what makes the honeycomb lattice structure of endocytic pits, is co-localized with adaptor proteins at membrane surfaces. It is thought that adaptor proteins are targeted to active cytoplasmic tail tyrosine residues of ligand-bond receptors such as FcγR's **(Pearse & Robinson, 1990)**. However, previous work in our lab may suggest that FcγRI can effectively endocytose without a tyrosine being present in its cytoplasmic region. Indeed, an absent cytoplasmic domain did not hampered FcγRI's endocytic ability **(Harrison et al., 1994)**.

Perhaps this adaptor recognition allows structural changes of the membrane, resulting in efficient particle engulfment. In the standard model, internalized particle remains surrounded by clathrin and an inner layer of adaptor proteins. By an unknown mechanism, prior to fusion to endocytic or lysozomal compartments both clathrin and adaptor proteins are removed.

Interestingly, the type of adaptor protein a receptor recruits determines the destination of the coated pit endocytosed in the cytoplasmic domain. For example the mannose-6-phosphate receptor in the plasma membrane binds the HA-1 adaptor protein; however, in the Golgi the same protein only associated with the HA-2 adaptor protein. In contrast, LDL and transferrin receptor associate with HA-1 in the Golgi and HA-2 when located on the plasma membrane **(Pearse & Robinson, 1990)**. While there is considerable knowledge about a crucial role for adaptors in internalization of ligand, the specific role adaptors play by linking individual receptors to clathrin, and then targeting the complex towards particular cytoplasmic domains remains unclear. Along those lines the unique adaptor proteins associated with FcγR's have not been elucidated.

5.3.3 Efficient Phagocytosis via ITAM Signalling Requires Tyrosine Kinases in COS-7 Cells.

In previous experiments conducted in the lab it was shown that for FcγRI and γ chain co-transfected COS-7 cells, internalisation was due to phagocytosis and was dependent on the recruitment of tyrosine kinases.

When treated with the tyrosine kinase inhibitor Genistein, FcγRI + γ chain co-tranfected COS-7 cells showed phagocytic ability reduced to the level of control cells. When similarly transfected cells were treated with cytochalasin-B, an F-actin polymerisation inhibitor, phagocytosis was blocked. F-actin polymerisation is required for pseudopodia engulfment of target particles. From Joe Hutchinson's results it can be determined that particle engulfment in transfected COS-7 cells is a phagocytic process that requires tyrosine kinases.

5.3.4 The Effects of Endogenous Tyrosine Kinases

Experiments show that co-transfections of FcγRI and signalling subunits can phagocytose. Since tyrosine kinases are required for this phagocytosis, it is clear that the endogenous COS-7 tyrosine kinases are able to interact with the ITAMs on these accessory molecules and initiate downstream signalling events. As mentioned before, an endogenous Fyn-like kinase has already been found in COS-7 cells.

In co-transfection experiments, the amount of tyrosine kinase that is expressed at threshold levels of receptors is hard to determine (as rosetting is not an indication). Indeed, for a true indication of the effect of added tyrosine kinases on phagocytosis, the experiments would have to be designed so that endogenous tyrosine kinases did not interfere. Ideally, it would be insightful to see which simian tyrosine kinases were expressed naturally in COS-7 cells by Northern analysis.

Potentially, before transfection began, cells could be treated with anti-sense cDNAs to the kinase in question. In this way, in the absence of endogenous tyrosine kinases, the effects on phagocytosis could be examined. For example, it would be interesting to see if anti-sense Fyn could block FcγRI/ζ phagocytic ability in COS-7 cells.

Another important note to make is that there are many different species of cDNAs involved in this phagocytosis test. Human receptors and signalling subunits transfected into a simian cell line, with cotransfected human and mouse tyrosine kinases. The effects of this combination of cDNAs in each signalling complex has to be taken into account.

5.3.5 mLyn and hFyn Tyrosine Kinase Were Co-transfected with Mutant Receptors FcγRI/γ, FcγRI/ζ, and FcγRI/II

These particular tyrosine kinases were chosen for several reasons. All Src-family N-terminal region (SH4 domain) has been defined as the location that allows tyrosine kinases to bind ITAM residues in non-aggregated receptor complexes **(Cambier, 1995)**. In particular, the SH4 domain present in Lyn and Fyn members has been found to mediate TCR (ζ-chain) and BCR (γ-chain) binding as well as enabling protein membrane attachment **(Gauen *et al.*, 1996)**.

5.3.6 SH4 Binding

Tyrosine binding occurs through the tyrosine kinase SH2 domain **(Howe & Weiss, 1995)**. However, both Lyn and Fyn SH4 domains bind an ITAM intermittent motif -DCSM- in non-phosphorylated resting receptors **(Clark *et al.*, 1994)**. When the BCR Igb subunit had its tyrosine intermittent motif, -QTAT- replaced with the -DCSM- motif, Fyn, Lyn, and Blk binding was enhanced post-activation **(Choquet *et al.*, 1994) (Cambier, 1995)**.

The N-terminal SH4 region of Lyn possesses the same 4 crucial residues found on the Fyn, namely Gly2, Cys3, Lys7, and Lys9. These residues allow it to bind ITAMs of the γ-chain prior to activation **(Amouri et al., 1997)**. The Gly2 and Cys3 residues not only enable BCR and TCR binding, but they also serve the dual function of myristoylation and palmitoylation respectively of these proteins **(Gauen *et al.*, 1996)**. The 2 basic lysines allow for direct binding to ζ-chain and γ-chain prior to activation **(Gauen et al., 1992)**.

5.3.7 Justification of Tyrosine Kinases Lyn and Fyn Used in Transfection Experiments

Although other src-family proteins have been implicated in TCR and BCR signalling, they do not bind directly to ζ-chain or γ-chain prior to activation. While all src-family proteins have Gly2 and Cys3 residues, they lack the 3rd important feature of SH4 domains, the 2 basic lysine residues at positions 7 & 9. This is important as only γ-chain and ζ-chain transmembrane and cytoplasmic domains are retained in the FcγRI/γ and FcγRI/ζ mutants.

Indeed the Lck tyrosine kinase has been shown to be more critical to TCR signalling than Fyn kinase. The SH2 domain of Lck in TCR was found essential to Lck association with ZAP-70 and ζ chain **(Straus *et al.*, 1996)**. Lck p56 has been shown repeatedly to be the TK necessary for zeta chain

activation in the TCR. However, Lck SH3 domains binds the CD4 and CD8 glycoproteins of the TCR in resting receptors. Lck does not possess the N-terminal motif necessary for direct ζ-chain binding and binds to the CD4 and CD8 components instead.

Multiple src-family kinases besides Lyn have been implicated in γ-chain mediated signalling. Hck and Lyn are found to associate with FcγRI-gamma chain complex in THP-1 human monocyte cell line. Hck, Blk, and Lyn proteins were found to increased their state of phosphorylation and kinase activity following receptor cross-linking **(Wang et al., 1994).(Duchemin & Anderson, 1997)(Choquet *et al.*, 1994) (Cambier, 1995).**

However, Lyn has been shown more critical to γ-chain signalling than Hck and Blk in previous studies. It was demonstrate that p53/56 Lyn associates with resting FcεRI in RBL-2H3 mucosal mast cells and upon activation a rapid increase Lyn kinase found associated with this receptor is observed 1 minute after stimulation, and a sharp increase in kinase activity is seen **(Field *et al.*, 1995)**. Lyn deficient B cells were also found unable to support signal transduction **(Johnson *et al.*, 1995)**. Hck and Blk tyrosine kinases also lacks a SH4 motif needed for direct γ-chain binding.

Lastly, evidence suggests that when FcγRII is stimulated on platelets, its ITAM tyrosines are rapidly phosphorylated by src-family proteins, with the src protein itself being strongly implicated **(Huang *et al.*, 1992).** However, the Src tyrosine kinase was not available for use in these experiments. Perhaps further COS-7 phagocytosis studies using Src TK would yield interesting results when cotransfected with FcγRI/II.

It was therefore decided that for these internalisation experiments, Fyn versus Lyn tyrosine kinases would be tested on their relative ability to aid γ-chain, ζ-chain, and FcγRII instigated phagocytosis. However, future studies may want to test the relative effects of Hck, Blk, Lck, and Src on mutant receptor phagocytosis.

5.3.8 Only the Effect of the Tyrosine Kinase mSyk on FcγRI/γ, FcγRI/ζ, and FcγRI/II Internalisation Was Very Significant

All cells cotransfected with mSyk and FcγRI + signalling domains showed increases in the average number of SRBCs internalised and also increases of the percent positive cells capable of phagocytosis. mSyk cotransfected cells have some individuals cells that have massive amounts of internalised SRBC. They also have many more cells that have internalised single SRBCs.

5.3.9 Pooling of Results on Tyrosine Kinases Lyn, Fyn, and Syk

The experiments conducted showed only slight differences in the tyrosine kinases recruited by distinct receptor ITAMs. However, it was observed that the non-src family kinase Syk had an overwhelming effect on phagocytosis. There are several reasons why perhaps the mLyn and hFyn tyrosine kinases are less effective at aiding phagocytosis than mSyk. As mentioned previously, it has been hypothesised that there is some form of negative feedback loop whereby endogenous tyrosine kinase expression of Fyn and Lyn is stopped when plasmid copy levels reach a threshold. Seemingly Syk remains unaffected by this cellular control mechanism.

This suggests that Syk may be the limiting factor in src-family tyrosine kinase regulation. When excess Syk is transcribed inside the cell, monitors pick up on the increases and relax regulation of src-family tyrosine kinase transcription.

It is also possible that an inhibitor, such as a phosphatase, is switched on at the transcription level in reaction to the high levels of tyrosine kinases introduced inside the cell. Interestingly, Syk family proteins lack an inhibitory carboxy terminal tyrosine. This location is the point of interaction on src-family kinases with regulatory phosphatases. Perhaps over-expression of tyrosine kinases enhances endogenous phosphatase production in an attempt to "put the brakes" on TK activity. While phosphatases inhibit src-family activity, it is unable to stop the downstream Syk signalling activity. This would explain why cotransfection with Lyn and Fyn did not dramatically affect PI while cotransfections with Syk did.

5.3.10 What the Syk Response Suggests About ITAM Mediated Phagocytosis

Originally it was thought that those cells which mediate phagocytosis are those cells which express high levels of receptor. There has been a noted discrepancy between the number of cells showing rosetting and those capable of phagocytosis. It was predicted that low level expression of receptor on the cell surface allowed for rosetting, but low level expression was sub-optimal for consequent internalisation of ligand. This would support Griffin and Silverstein's "zipper " model of phagocytosis. Expressed receptors line the

phagocytic pocket and advance around a target particle until receptors bind all of its surface and the particle becomes engulfed.

However, the dramatic increase in PI which was observed for Syk cotransfected cells does not support this "zipper" model. The effects of Syk cannot be attributed to its ability to upregulate expression of its co-transfected receptor. If anything the COS-7 machinery would be less efficient at receptor expression, taxed by multiple vectors. As described in the results, the percent positive cells that have internalised 10 or more SRBCs approximate the number of total percent positive cells in an identical mSyk$^-$ plate. It was suggested that Syk somehow decreases the threshold of internalisation, so cells that normally internalise small numbers of SRBCs begin to internalise large numbers, and many cells that previously internalised no SRBCs at all are now capable of some internalisation. This supports the "trigger" all or nothing model of internalisation moreso that the "zipper" model **(Griffin and Silverstein, 1975)(Silverstein *et al.*, 1989)**.

This brings back into the spotlight one of the main points of contention in Fc signalling. Is the Syk family protein recruited to the biphosphorylated ITAM, or is it alternatively recruited to membrane bound, receptor associated src-family kinases. As these COS-7 studies show, src-family TKs do not need to be over-expressed to allow transfected Syk to initiate extremely efficient phagocytosis.

The pivotal role of syk-family kinases and not src-family kinases in phagocytosis models has been further supported by other studies. In 1992 Kolanus found that chimeras of CD61 extracellular and Syk intracellular domains are sufficient to transduce signalling, while CD16 extracellular and src-family intracellular are not **(Kolanus *et al.*, 1992)**. Chimeric receptor with cytoplasmic domains consisting exclusively of Syk TK sequences were recently found capable of initiating phagocytosis in COS cells **(Greenberg *et al.*, 1996)**. Using a stem looped anti-syk inhibitor it was found that in the absence of Syk, FcγRII mediated phagocytosis was not able to take place, although normal phosphorylation of FcγRII ITAM tyrosines was not disrupted **(Matsuda *et al.*, 1996)**. And in platelets FcγRII mediated signalling requires receptor association with Syk **(Chacko *et al.*, 1994)**. Lastly, cross-linking of the FcgammaRIIIA on pulmonary phagocytic macrophages stimulated a four fold increase in γ-subunit associated Syk kinase activity as assessed by autophosphorylation **(Darby *et al.*, 1994)**. The importance of Syk family recruitment in receptor signalling mechanisms is becoming universally accepted.

5.3.11 To Further Study FcγRI/ζ Internalisation

To continue studies on FcγRI/ζ signalling several lines of investigation can be followed. It would be interesting to see whether it is a specific ITAM of the 3 zeta chain ITAMs that is most responsible for cell signalling, or whether it is the specific distance of any of the three ITAMs from the membrane that is important (so that membrane attached tyrosine kinases can bind to it).

Already other experiments have examined the sequence requirements of ζ needed to induce cytolysis and free calcium ion mobilization in CTL's (cytotoxic T cells). As few as 18 amino acid had to be present in the tail region of CD8+/ζ fusion proteins for these post-activation events to proceed (**Romeo *et al.*, 1992**). These chimeras contained only the first ITAM of ζ chain compared to the three of the wild-type chain. The one ITAM chimera showed the same activation events as the wild-type but were 8 fold less active than the full length ζ chain chimeras (**Romeo *et al.*, 1992**).

These two questions could be addressed experimentally as follows. To the FcγRI/ζ mutant, alanine mutation could be used to selectively "knock out" tyrosines apart from ζ1, ζ2, and then ζ3 in turn. In this way the effect of distance can be measured on FcγRI/ζ's ability to phagocytose in the COS-7 model.

Secondly, FcγR/ζ1, FcγRI/ζ2, and then FcγRI/ζ3 can be constructed so that each ITAM is equidistance from the cell membrane. Phagocytosis experiments will determine whether each ITAM is equally as effective at phagocytosis. Also, if phagocytic potential is decreased when only one of three ITAMs is present in these mutants, then the three intrinsic ζ chain ITAMs must be thought to contribute to signal amplification.

The FcγRI/ζ1 was in the process of being constructed (see results). It should be relatively easy to construct, as tail ζ2 and ζ3 can be removed by vector digests and the FcγRI + ζ1 +CDM can be religated together following Klenow removal of 5'overhangs. The ζ2 and ζ3 mutants were to be constructed so that they were an identical distance from the membrane as the ζ1 mutant. The ζ2 and ζ3 were to be constructed by introducing specific restriction sites at intermittent sequences around the specific motif by PCR. Digests and religations were to be designed to then realign the individual ITAMs at the desired location.

5.4 Discussion Summary

The first set of results investigated the differences between signal transduction events within the cell for FcγRI + γ chain and FcγRII on U937 monocyte-like cells. The focus was to define the pattern of tyrosine kinase phosphorylation after FcγR cross-linking in differentiated and then undifferentiated monocytes. IFNγ treated U937 cells have FcγRI and γ chain upregulation, while DBC treated U937 cells have FcγRII upregulation.

A differential pattern was observed for IFNγ and DBC treated cells. Firstly, while IFNγ cells show peak Syk activation 5-7 minutes post activation that is still high 30 minutes later, DBC cells show an immediate increase in Syk recruitment following activation that is tailing off by the 30 minute mark. Secondly, blots of post-activation lysates of the two cell types were probed with anti-phosphotyrosine antibody. This revealed a strong band of 110kDa on both IFNγ and DBC blots. However, the identity of a 50kDa band found exclusively on the DBC blot remains unknown. Reprobing with a general anti-src antibody did not help elucidate the bands identity. Thirdly, experiments designed to test whether or not the two cells types display alternative NO_2 release patterns post-activation were unsuccessful, so this has yet to be determined.

A second set of experiments was designed to examine differential patterns of upstream tyrosine kinase recruitment of the various FcγR associated signalling domains. Firstly, a chimeric receptor was made by fusion of the extracellular region of FcγRI to the transmembrane and cytoplasmic tail of zeta chain. The novel FcγRI-ζ was capable of rosetting and phagocytosis, indicative of an intact and functioning signalling domain. This chimeric receptor was then compared to other previously constructed mutants, FcγRI-γ and FcγRI-II for its ability to phagocytose sheep red blood cells (SRBC) in a well established phagocytosis experiment using COS-7 cells (a simian kidney fibroblast cell line).

Firstly, baseline ability of the various mutants to internalise sheep red blood cells was established. These internalisation experiments were dependent on the recruitment of endogenous COS-7 tyrosine kinases, as phagocytosis is a tyrosine kinase dependent event. FcγRI-γ, FcγRI-II, and FcγRI-ζ all demonstrated relatively equivalent phagocytic ability in the COS-7 cell system.

Subsequently, select tyrosine kinases were co-transfected with mutant receptors with distinct signalling domains. It was hoped that distinct ITAMs would demonstrate an alternative dependency for specific tyrosine kinases. There was no dramatic difference between tyrosine kinases required

by each subunit. However, it was discovered that only the effect of the tyrosine kinase mSyk on FcγRI/γ, FcγRI/ζ, and FcγRI/II internalisation was significant. Co-transfections of the mutant receptors with tyrosine kinases mLyn and hFyn had only a slight effect on phagocytic levels.

These results suggest that Syk may be the limiting factor in src-family tyrosine kinase regulation. Co-transfections with Syk seem to decreases the threshold of internalisation, by what seems to be an all or nothing event. Finally, this pivotal role of syk-family kinases supports the Griffin and Silverstein "trigger" model of internalisation.

References

Abo, T., Tilden, A. B., Balch, C. M., and et, al. (1984). Ethnic differences in the lymphocyte proliferative response induced by a murine IgG1 antibody, Leu-4, to the T3 molecule. **Journal of Experimental Medicine 160**, 303-309.

Agarwal, A., Salem, P., and Robbins, K.C. (1993). Involvement of $p72^{syk}$, a Protein-Tyrosine Kinase, in Fcγ Receptor Signalling. **The Journal of Biological Chemistry 268**, 15900-15905.

Alland, L., Peseckis, S.M., Atherton, R.E., Berthiaume, L., Resh, M.D. (1994). Dual Myristylation and Palmitylation of Src Family Member p59(fyn) Affects Subcellular-localization. **Journal of Biological Chemistry 269**, 16701-16705.

Allen, J. M., and Seed, B. (1989). Isolation and expression of functional high-affinity Fc receptor complementary DNAs. **Science 243**, 378-381.

Amigorena, S., Salamero, J., Davoust, J., Fridman, W. H., and Bonnerot, C. (1992). Tyrosine-containing motif that transduces cell activation signals also determines internalization and antigen presentation via type III receptors for IgG. **Nature 358**, 337-341.

Amoui, M., Draberova, L., Tolar, P., and Draber, P. (1997). Direct interaction of Syk and Lyn protein tyrosine kinases in rat basophilic leukemia cells activated via type I Fcepsilon receptors. **European Journal of Immunology 27**, 321-328.

Anderson, C. L., Shen, L., Eicher, D. M., Wewers, M. D., and Gill, J. K. (1990a). Phagocytosis mediated by three distinct Fcgamma receptor classes on human leukocytes. **Journal of Experimental Medicine 171**, 1333-1345.

Anderson, P., Caligiuri, M., Obrien, C., Manley, T., Ritz, J., and Schlossman, S. F. (1990b). Fc(gamma) receptor type III (CD16) is included in the zeta NK receptor complex expressed by human natural killer cells. **Proceedings of the National Academy of Sciences of the United States of America 87**, 2274-2278.

Aoki, Y., Kim, Y., Stillwell, R., Kim, T.J., and Pillai, S. (1995). The SH2 Domains of Src Family Kinases Associate with Syk. **The Journal of Biological Chemistry 270**, 15658-15663.

Ashman, R.F., Peckman, D., and Stunz, L.L. (1996). Fc Receptor Off-Signal in the B Cell Involves Apoptosis. **The Journal of Immunology 157**, 5-11.

Azzoni, L., Kamoun, M., Salcedo, T.W., Kanakara. J.P., and Perussia, B. (1992). Stimulation of Fc-gamma-RIIIa Results in Phospholipase C-gamma-1 Tyrosine Phosphorylation and p56(lck) Activation. **Journal of Experimental Medicine 176**, 1745-1750.

Beaven, M.A. (1996). Calcium Signaling - Sphingosine Kinase Versus Phospholipase-C. **Current Biology 6**, 798-801.

Bianca, V.D., Dusi, S., Nadalini, K.A., Donini, M., Rossi, F. (1995). Role of 55- and 75-kDa TNF Receptors in the Potentiation of Fc-Mediated Phagocytosis in Human Neutrophils. **Biochemical and Biophysical Research Communications 214**, 44-50.

Blank, U., Ra, C., Miller, L., White, K., Metzger, H., and Kinet, J. P. (1989). Complete structure and expression in transfected cells of high affinity IgE receptor. **Nature 337**, 187-189.

Bokoch, G.M. (1995). Regulation of the Phagocyte Respiratory Burst by Small GTP-binding Proteins. **Trends in Cell Biology 5**, 109-113.

Bonnerot, C., and Amigorena, S. (1993). Murine Low-Affinity Receptors for the Fc Portion of IgG. Roles in Cell Activation and Ligand Internalization. **Receptors and Channels 1**, 73-79.

Bubien, J.K., Zhou, L.J., Bell. P.D., Frizzell, R.A., and Tedder, T.F. (1993). Transfection of the CD20 Cell-surface Molecule into Ectopic Cell-types Generates a Ca^{2+} Conductance Found Constitutively in B-lymphocytes. **Journal of Cell Biology 121**, 1121-1132.

Burgess, J.K., Lindeman, R., Chesterman, C.N., and Chong, B.H. (1995) Single Amino Acid Mutation of the Fcγ Receptor is Associated with the Development of Herapin-Induced Thrombocytopenia. **British Journal of Haematology 91,** 761-766.

Cambier, J. C. (1995a). Antigen and Fc receptor signaling the awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). **Journal of Immunology 155,** 3281-3285.

Ceuppens, J. L., Baroja, M. L., Van Vaeck, F., and Anderson, C. L. (1988). Defect in the membrane expression of high affinity 72-kD Fc gamma receptors on phagocytic cells in four healthy subjects. **Journal of Clinical Investigation 82,** 571-578.

Chacko, G. W., Brandt, J. T., Coggeshall, K. M., and Anderson, C. L. (1996). Phosphoinositide 3-kinase and p72(syk) noncovalently associate with the low affinity Fcgamma receptor on human platelets through an immunoreceptor tyrosine-based activation motif: Reconstitution with synthetic phosphopeptides. **Journal of Biological Chemistry 271,** 10775-10781.

Chacko, G. W., Duchemin, A. M., Coggeshall, K. M., Osborne, J. M., Brandt, J. T., and Anderson, C. L. (1994). Clustering of the platelet Fcgamma receptor induces noncovalent association with the tyrosine kinase p72(syk). **Journal of Biological Chemistry 269,** 32435-32440.

Chan, A.C., van Oers, N.S.C., Tran, A., Turka, L., Law, C., Ryan, J.C., Clark, E.A., and Weiss, A. (1994). Differential Expression of ZAP-70 and Syk Protein Tyrosine Kinases, and the Role of This Family of Protein Tyrosine Kinases in TCR Signalling. **Journal of Immunology 152,** 4758-4766.

Choi O.H., Kim, J.H., and Kinet, J.P. (1996). Calcium Mobilization Via Sphingosine Kinase in Signalling by the FcεRI Antigen Receptor. **Nature 380,** 634-636.

Choquet, D., Ku, G., Cassard, S., Malissen, B., Korn, H., Fridman, W.H., Bonnerot, C. (1994). Separate Patterns of Calcium Signaling Triggered Through 2 Components of the B-lymphocyte Antigen Receptor. **Biophysical Journal 66,** 151

Clark, M. R., Campbell, K. S., Kazlauskas, A., Johnson, S. A., Hertz, M., Potter, T. A., Pleiman, C., and Cambier, J. C. (1992). The B cell antigen receptor complex: Association of Ig-alpha and Ig- beta with distinct cytoplasmic effectors. **Science 258**, 123-126.

Clark, M.R., Johnson, S.A., and Cambier, J.C. (1994). Analysis of Ig-alpha Tyrosine Kinase Interaction Reveals 2 Levels of Binding-specificity and Tyrosine-phosphorylated Ig-alpha Stimulation of Fyn Activity. **EMBO Journal 13**, 1911-1919.

Daeron, M., Malbec, O., Bonnerot, C., Latour, S., Segal, D. M., and Fridman, W. H. (1994). Tyrosine-containing activation motif-dependent phagocytosis in mast cells. **Journal of Immunology 152**, 783-792.

Darby, C., Geahlen, R.L., and Schreiber, A.D. (1994). Stimulation of Macrophage Fc-gamma-RIIIa Activates the Receptor- associated Protein-tyrosine Kinase Syk and Induces Phosphorylation of Multiple Proteins Including p95vav and p62 Gap-associated Protein. **Journal of Immunology 152**, 5429-5437

Davis, W., Halliwell, E.L., Sage, S.O., and Allen, J.M. (1995). Increased Capacity for Store Regulated Calcium Influx in U937 Cells Differentiated by Treatment with Dibutyryl cAMP. **Cell Calcium 17**, 345-353.

Davis, W., Harrison, P. T., Hutchinson, M. J., and Allen, J. M. (1995). Two distinct regions of FcgammaRI initiate separate signalling pathways involved in endocytosis and phagocytosis. **EMBO Journal 14**, 432-441.

Davis, W., Sage, S.O., and Allen, J.M. (1994). Cytosolic Calcium Elevation in Response to Fc Receptor Cross-linking in Undifferentiated and Differentiated U937 Cells. **Cell Calcium 16**, 29-36.

Duchemin, A. M., and Anderson, C. L. (1997). Association of non-receptor protein tyrosine kinases with the Fc gamma RI/gamma-chain complex in monocytic cells. **Journal Of Immunology 158**, 865-871.

Duchemin, A. M., Ernst, L. K., and Anderson, C. L. (1994). Clustering of the high affinity Fc receptor for immunoglobulin G (FcgammaRI) results in phosphorylation of its associated gamma-chain. **Journal of Biological Chemistry 269**, 12111-12117.

Dwek, R.A., Lellouch, A.C., and Wormald, M.R. (1995). Glycobiology:'The Function of Sugar in the IgG Molecule'. **Journal of Anatomy 187**, 279-292.

Ernst, L. K., Duchemin, A. M., and Anderson, C. L. (1993). Association of the high-affinity receptor for IgG (FcgammaRI) with the gamma subunit of the IgE receptor. **Proceedings of the National Academy of Sciences of the United States of America 90**, 6023-6027.

Ernst, L. K., Van de Winkel, J. G. J., Chiu, I. M., and Anderson, C. L. (1992). Three genes for the human high affinity Fc receptor for IgG (FcgammaRI) encode four distinct transcription products. **Journal of Biological Chemistry 267**, 15692-15700.

Field, K.A., Holowka, D., and Baird, B. (1995). FcεRI-mediated Recruitment of p53/56^{lyn} to Detergent-resistant Membrane Domains Accompanies Cellular Signalling. **Proceedings of the National Academy of Sciences of the United States of America 92**, 9201-9205.

Frank, S.J., Niklinska, B.B., Orloff, D.G., Mercep, M., Ashwell, J.D., Klausner, R.D. (1990). Structural Mutations of the T Cell Receptor ζ Chain and Its Role in T Cell Activation. **Science 247**, 174-177.

Fridman, W.H., Bonnerot, C., Daeron, M., Amigorena, S., Teillaud, J.L., Sautes, C. (1992) Structural Bases of Fc-gamma Receptor Functions. **Immunological Reviews 125**, 49-76.

Gauen, L. K. T., Linder, M.E., and Shaw, A.S. (1996). Mutiple features of the p59^{fyn} src Homology 4 Domain Define a Motif for Immune-Receptor Tyrosine-based Activation Motif (ITAM) Binding and for Plasma Membrane Localization. The **Journal of Cell Biology 133**, 1007-1015.

Gauen, L. K. T., Zhu, Y., Letourneur, F., Hu, Q., Bolen, J. B., Matis, L. A., Klausner, R. D., and Shaw, A. S. (1994). Interactions of p59(fyn) and ZAP-70

with T-cell receptor activation motifs: Defining the nature of a signalling motif. **Molecular and Cellular Biology 14**, 3729-3741.

Ghazizadeh, S., Bolen, J. B., and Fleit, H. B. (1994b). Physical and functional association of Src-related protein tyrosine kinases with FcgammaRII in monocytic THP-1 cells. **Journal of Biological Chemistry 269**, 8878-8884.

Ghazizadeh, S., and Fleit, H. B. (1994a). Tyrosine phosphorylation provides an obligatory early signal for FcgammaRII- mediated endocytosis in the monocytic cell line THP-1. **Journal of Immunology 152**, 30-41.

Gluzman, Y. (1981). Sv40-Transformed Simian Cells Support the Replication Of Early Sv40 Mutants. **Cell 23**, 175-182.

Greenberg, S. (1995). Signal transduction of phagocytosis. **Trends in Cell Biology 5**, 93-99.

Greenberg, S., Burridge, K., and Silverstein, S. C. (1990). Colocalization of F-actin and talin during Fc receptor-mediated phagocytosis in mouse macrophages. **Journal of Experimental Medicine 172**, 1853-1856.

Greenberg, S., Chang, P., and Silverstein, S. C. (1994). Tyrosine Phosphorylation Of the Gamma-Subunit Of Fc(Gamma)-Receptors, P72(Syk), and Paxillin During Fc-Receptor-Mediated Phagocytosis In Macrophages. **Journal Of Biological Chemistry 269**, 3897-3902.

Greenberg, S., Chang, P., Wang, D. C., Xavier, R., and Seeds, B. (1996). Clustered syk tyrosine kinase domains trigger phagocytosis. **Proceedings of the National Academy of Sciences of the United States of America 93**, 1103-1107.

Griffin, F. M. J., Griffin, J. A., Leider, J. E., and Silverstein, S. C. (1975). Studies on the mechanism of Phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. **Journal of Experimental Medicine 142**, 1263-1282.

Gauen, I.K.T., Letourneur, F., Kong, A.N.T., Samelson, L.E., Klausner, R.E, and Shaw, A.S. (1993). The p59(Fyn) Tyrosine Kinase Associates with CD3 Epsilon Cytoplasmic Residues Required for T-cell Activation. **Journal of Immunology 150**, 269.

Gupta, S., Weiss, A., Kumar, G., Wang, S., and Nel, A. (1994). The T-cell Antigen Receptor Utilizes Lck, Raf-1, and MEK-1 for Activating Mitogen-activated Protein Kinase. **The Journal of Biological Chemistry 269**, 17349-17357.

Guyre, P. M., Graziano, R. F., Vance, B. A., Morganelli, P. M., and Fanger, M. W. (1989). Monoclonal antibodies that bind to distinct epitopes on FcgammaRI are able to trigger receptor function. **Journal of Immunology 143**, 1650-1655.

Haas , M.D., Kleijer, M., van Zwieten, R., Roos, D., and von dem Borne, A.E.G.Kr. (1995). Neutrophil FcγRIIIb Deficiency, Nature, and Clinical Consequences: A Study of 21 Individuals From 14 Families. **Blood 86**, 2403-2413.

Hall, C. G., Sancho, J., and Terhorst, C. (1993). Reconstitution of T cell receptor zeta-mediated calcium mobilization in nonlymphoid cells. **Science 261**, 915-918.

Hanke, J.H., Gardner, J.P., Dow, R.L., Changelian, P.S., Brissette, W.H., Weringer, E.J., Pollok, B.A., and Connelly, P.A. (1996). Discovery of a Novel, Potent, and Src Family-selective Tyrosine Kinase Inhibitor. **The Journal of Biological Chemistry 271**, 695-701.

Harrison, P. T. (1996). Protein: Protein interactions in the lipid bilayer (Review). **Molecular Membrane Biology 13**, 67-79.

Harrison, P. T., and Allen, J. M. (1997). High affinity IgG binding by FcgRI (CD64) is modulated by two distinct IgSF domains and the transmembrane domain of the receptor. **Submitted for publication.**

Harrison, P. T., Bjorkhaug, L., Hutchinson, M. J., and Allen, J. M. (1995). The Interaction Between Human Fc-Gamma-Ri and the Gamma-Chain Is

Mediated Solely Via the 21 Amino-Acid Transmembrane Domain Of Fc-Gamma-Ri. **Molecular Membrane Biology 12**, 309-312.

Harrison, P. T., Davis, W., and Allen, J. M. (1993). Stimulation of endocytosis by antibody cross-linking of the human high affinity receptor for IgG in COS cells is independent of the cytoplasmic and transmembrane domains. **Biochemical Society Transactions 21**, 335s.

Harrison, P. T., Davis, W., Norman, J. C., Hockaday, A. R., and Allen, J. M. (1994b). Binding of monomeric immunoglobulin G triggers FcgammaRI-mediated endocytosis. **Journal of Biological Chemistry 269**, 24396-24402.

Harrison, P. T., Hutchinson, M. J., and Allen, J. M. (1994a). A convenient method for the construction and expression of GPI- anchored proteins. **Nucleic Acids Research 22**, 3813-3814.

Henson, P. M., Zanolari, B., Schwartzman, N. A., and Hong, S. R. (1978). Title unknown. **Journal of Immunology 121**, 851-.

Hitt, A. L., Hartwig, J. H., and Luna, E. J. (1994). Ponticulin is the major high affinity link between the plasma membrane and the cortical actin network in Dictyostelium. **Journal of Cell Biology 126**, 1433-1444.

Howe, L.R., and Weiss, A. (1995). Mutiple Kinases Mediate T Cell Receptor Signalling. **Trends In Biological Science 20**, 59-64.

Huang, M. M., Indik, Z., Brass, L. F., Hoxie, J. A., Schreiber, A. D., and Brugge, J. S. (1992). Activation of FcgammaRII induces tyrosine phosphorylation of multiple proteins including FcgammaRII. **Journal of Biological Chemistry 267**, 5467-5473.

Hulett, M. D., and Hogarth, P. M. (1994). Molecular basis of Fc receptor function. **Advances in Immunology 57**, 1-127.

Hutchinson, M. J., and Allen, J. M. (1996). The functional role of the gamma-chain cysteine in FcgammaRI-mediated phagocytosis using co-transfected COS-7 cells. **Biochemical Society Transactions 24**, 114s.

Hutchinson, M. J., Harrison, P. T., and Allen, J. M. (1995a). Transmembrane association with gamma-chain is required for Fc(gamma)RI-mediated phatocytosis in transfected COS cells. **Biochemical Society Transactions 23**, 121s.

Hutchinson, M. J., Harrison, P. T., Floto, R. A., and Allen, J. M. (1995b). Fcgamma receptor-mediated phagocytosis requires tyrosine kinase activity and is ligand independent. **European Journal of Immunology 25**, 481-487.

Indik, Z. K., Park, J. G., Hunter, S., Mantaring, M., and Schreiber, A. D. (1995a). Molecular dissection of Fcgamma receptor-mediated phagocytosis. **Immunology Letters 44**, 133-138.

Indik, Z. K., Xiao Qing, P., Huang, M. M., McKenzie, S. E., Levinson, A. I., and Schreiber, A. D. (1994a). Insertion of cytoplasmic tyrosine sequences into the nonphagocytic receptor FcgammaRIIB establishes phagocytic function. **Blood 83**, 2072-2080.

Irving, B.A., and Weiss, A. (1991). The Cytoplasmic Domain of the T Cell Receptor Zeta Chain Is Sufficient to Couple to Receptor-Associated Signal Transduction Pathways. **Cell 64**, 891-901.

Johnson, S.A., Pleiman, C.M., Pao, L., Schneringer, J., Hippen, K., and Cambier, J.C. (1995). Phosphorylated Immunoreceptor Signalling Motifs (ITAMs) Exhibit Unique Abilities to Bind and Activate Lyn and Syk Tyrosine Kinases. **The Journal of Immunology 155**, 4596-4603.

Katzav, S., Sutherland, M., Packham, G., Yi, T., and Weiss, A. (1994). The Protein Tyrosine Kinase ZAP-70 Can Associate with the SH2 Domain of Proto-Vav. **The Journal of Biological Chemistry 269**, 32579-32585.

Kinet, J.P. (1989). Antibody-Cell Interactions: Fc Receptors. **Cell 57**, 351-354.

Kinet, J. P. (1996). Personal communication.

Klausner, R.D., and Samelson, L.E. (1991). T Cell Antigen Receptor Activation Pathways: The Tyrosine Kinase Connection. **Cell 64**, 875-878

Koegl, M., Zlatkine, P., Ley, S.C., Courtneidge, S.A., and Magee, A.I. (1994). Palmitoylation of Multiple Src-family Kinases at a Homologous N-terminal Motif. **Biochemical Journal 303**, 749-753.

Kolanus, W., Romeo, C., and Seed, B. (1993). T cell activation by clustered tyrosine kinases. **Cell 74**, 171-183.

Kurosaki, T., Gander, I., and Ravetch, J. V. (1991). A subunit common to an IgG Fc receptor and the T-cell receptor mediates assembly through different interactions. **Proceedings of the National Academy of Sciences of the United States of America 88**, 3837-3841.

Kurosaki, T., Takata, M., Yamanashi, Y., Inazu, T., Taniguchi, T., Yamamoto, T., and Yamamura, H. (1994). Syk activation by the Src-family tyrosine kinase in the B cell receptor signaling. **Journal of Experimental Medicine 179**, 1725-1729.

Küster, H., Thompson, H., and Kinet, J. P. (1990). Characterization and expression of the gene for the human Fc receptor gamma subunit. Definition of a new gene family. **Journal of Biological Chemistry 265**, 6448-6452.

Lanier, L. L., Yu, G., and Phillips, J. H. (1989). Co-association of CD3zeta with a receptor (CD16) for IgG Fc on human natural killer cells. **Nature 342**, 803-805.

Letourneur, O., Kennedy, I. C. S., Brini, A. T., Ortaldo, J. R., Oshea, J. J., and Kinet, J. P. (1991). Characterization of the family of dimers associated with Fc receptors (FcepsilonRI and FcgammaRIII). **Journal of Immunology 147**, 2652-2656.

Lin, C. T., Shen, Z., Boros, P., and Unkeless, J. C. (1994). Fc receptor-mediated signal transduction. **Journal of Clinical Immunology 14**, 1-13.

Liscovitch, M., Cantley, L.C. (1994). Lipid 2nd Messengers. **Cell 77**, 329-334.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982a). Molecular Cloning: A Laboratory Manual, 1st Edn. Edition. Page 388.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982b). Molecular Cloning: A Laboratory Manual, 1st Edn. Edition. Pages 368-369.

Matsuda, M., Park, J. G., Wang, D. C., Hunter, S., Chien, P., and Schreiber, A. D. (1996). Abrogation of the Fcgamma receptor IIA-mediated phagocytic signal by stem- loop Syk antisense oligonucleotides. **Molecular Biology of the Cell 7**, 1095-1106.

McCarthy-Troke, M. H., Harrison, P. T., Campbell, I., and Allen, J. M. (1996). Scanning Alanine Mutagenesis Of the 2nd Ig Domain Of Fc-Gamma-Ri Indicates Critical Residues For Ligand Recognition. **Immunology 89**, Ff376-Ff376.

Miller, L., Alber, G., VarinBlank, N., Ludowyke, R., and Metzger, H. (1990). Transmembrane signaling in P815 mastocytoma cells by transfected IgE receptors. **Journal of Biological Chemistry 265**, 12444-12453.

Morton, H. C., Van den HerikOudijk, I. E., Vossebeld, P., Snijders, A., Verhoeven, A. J., Capel, P. J. A., and Van de Winkel, J. G. J. (1995). Functional association between the human myeloid immunoglobulin A Fc receptor (CD89) and FcR gamma chain. Molecular basis for CD89/FcR gamma chain association. **Journal of Biological Chemistry 270**, 29781-29787.

Mustelin, T., and Burn, P. (1993). Regulation of Src Family Tyrosine Kinases in Lymphocytes. **Trends in Biochemical Sciences 18**, 215-220.

Mustelin, T., Coggeshall, K.M., and Altman, A. (1989). Rapid Activation of the T-cell Tyrosine Protein-kinase pp56lck by the CD45 Phosphotyrosine

Phosphatase. **Proceedings of the National Academy of Sciences of the United States of America 86,** 6302-6306.

Odin, J. A., Edberg, J. C., Painter, C. J., Kimberly, R. P., and Unkeless, J. C. (1991). Regulation of phagocytosis and (Ca2+)(i) flux by distinct regions of an Fc receptor. **Science 254,** 1785-1788.

Orloff, D. G., Ra, C., Frank, S. J., Klausner, R. D., and Kinet, J. P. (1990). Family of disulphide-linked dimers containing the zeta and eta chains of the T-cell receptor and the gamma chain of Fc receptors. **Nature 347,** 189-191.

Ortega, E. (1995). How Do Multichain Immune Recognition Receptors Signal? A Structural Hypothesis. **Molecular Immunology 32,** 941-945.

Osman, N., Kozak, C.A., Mckenzie, I.F.C., Hogarth, P.M. (1992) Structure and Mapping of the Gene Encoding Mouse High-affinity Fc-gamma-RI and Chromosomal Location of the Human Fc-gamma-RI Gene. **Journal of Immunology 148,** 1570-1575

Paolini, R., Jouvin, M. H., and Kinet, J. P. (1991). Phosphorylation and dephosphorylation of the high-affinity receptor for immunoglobulin E immediately after receptor engagement and disengagement. **Nature 353,** 855-858.

Parolini, I., Sargiacomo, M., Lisanti, M.P., and Peschle, C. (1996). Signal Transduction and Glycophosphatidylinositol-Linked Proteins (LYN, LCK, CD4, CD45, G Proteins, and CD55) Selectively Localize in Triton-Insoluble Plasma Membrane Domains of Human Leukemic Cell Lines and Normal Granulocytes. **Blood 87,** 3783-3794.

Park, J. G., and Schreiber, A. D. (1995). Determinants of the phagocytic signal mediated by the type IIIA Fcgamma receptor, FcgammaRIIIA: Sequence requirements and interaction with protein- tyrosine kinases. **Proceedings of the National Academy of Sciences of the United States of America 92,** 7381-7385.

Pearse, R.N., Feinman, R., and Ravetch, J.V. (1991). Characterization of the Promoter of the Human Gene Encoding the High-affinity IgG Receptor -

transcriptional Induction by Gamma-interferon is Mediated Through Common DNA Response Elements. **Proceedings of the National Academy of Sciences of the United States of America 88**, 11305-11309.

Pearse, B.M.F., and Robinson, M.S. (1990). Clathrin, Adapters, and Sorting. **Annual Review of Cell Biology 6**, 151-171.

Pfefferkorn, L.C., and Swink, S.L. (1996). Intracluster Restriction of Fc Receptor γ-Chain Tyrosine Phosphorylation Subverted by a Protein-tyrosine Phasphatase Inhibitor. **The Journal of Biological Chemistry 271**, 11099-11105.

Pleiman, C.M., Chien, N.C., and Cambier, J.C. (1994). Point Mutations Define a mIgm Transmembrane Region Motif That Determines Intersubunit Signal-Transduction in the Antigen Receptor. **Journal of Immunology 152**, 2837-2844.

Pribluda, V. S., and Metzger, H. (1992). Transmembrane signaling by the high-affinity IgE receptor on membrane preparations. **Proceedings of the National Academy of Sciences of the United States of America 89**, 11446-11450.

Pribluda, V. S., Pribluda, C., and Metzger, H. (1994). Transphosphorylation as the mechanism by which the high-affinity receptor for IgE is phosphorylated upon aggregation. **Proceedings of the National Academy of Sciences of the United States of America 91**, 11246-11250.

Raghavan, M., Wang, Y., and Bjorkman, P.J. (1995). Effects of Receptor Dimerization on the Interaction Between the Class I Major Histocompatibility Complex-Related Fc Receptor and IgG. **Proceedings of the National Academy of Sciences of the United States of America 92**, 11200-11204.

Rankin, B.M., Yocum, S.A., Mittler, R.S., and Kiener, P.A. (1993). Stimulation of Tyrosine Phosphorylation and Calcium Mobilization by Fcγ Receptor Cross-linking. **The Journal of Immunology 150**, 605-616.

Ravetch, J. V. (1994). Fc receptors: Rubor redux. **Cell 78**, 553-560.

Ravetch, J. V., and Kinet, J. P. (1991). Fc receptors. **Annual Review of Immunology 9**, 457-492.

Ravetch, J.V., and Perussia, B. (1989). Alternative Membrane Forms of Fc-gamma-RIII(CD16) on Human Natural-killer Cells and Neutrophils - Cell Type-specific Expression of 2 Genes that Differ in Single Nucleotide Substitutions. **Journal of Experimental Medicine 170**, 481-497

Reth, M. (1989). Antigen Receptor Tail Clue. **Nature 338**, 383-384.

Romeo, C., Amiot, M., and Seed, B. (1992). Sequence requirements for induction of cytolysis by the T cell antigen/Fc receptor zeta chain. **Cell 68**, 889-897.

Romeo, C., and Seed, B. (1991). Cellular Immunity to HIV Activated by CD4 Fused to T Cell or Fc Receptor Polypeptides. **Cell 64**, 1037-1046.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 1st Edn. Edition, **Volume 3**. A11-12.

Samuelson, L.E., and Klausner, R.D. (1992). Tyrosine Kinases and Tyrosine-based Activation Motifs. **The Journal of Biological Chemistry 267**, 24913-24916.

Scholl, P. R., and Geha, R. S. (1993). Physical association between the high-affinity IgG receptor (FcgammaRI) and the gamma subunit of the high-affinity IgE receptor (FcepsilonRIgamma). **Proceedings of the National Academy of Sciences of the United States of America 90**, 8847-8850.

Seed, B., and Aruffo, A. (1987a). Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. **Proceedings of the National Academy of Sciences of the United States of America 84**, 3365-3369.

Segal, A.W, Abo, A. (1993). The Biochemical Basis of the Nadph Oxidase of Phagocytes. **Trends in Biochemical Sciences 18**, 43-47

Sheth, B., Dransfield, I., Partridge, L.J., Barker, M.D., Burton, D.R. (1988). Dibutyryl Cyclic-amp Stimulation of a Monocyte-like Cell-line, U937 - a

Model for Monocyte Chemotaxis and Fc Receptor-related Functions. **Immunology 63**, 483-490

Shiue, L., Zoller, M. J., and Brugge, J. S. (1995). Syk is activated by phosphotyrosine-containing peptides representing the tyrosine-based activation motifs of the high affinity receptor for IgE. **Journal of Biological Chemistry 270**, 10498-10502.

Sieh, M., Bolen, J.B., and Weiss, A. (1993). CD45 Specifically Modulates Binding of Lck to a Phosphopeptide Encompassing the Negative Regulatory Tyrosine of Lck. **The EMBO Journal 12**, 315-321.

Silverstein, S. C., Greenberg, S., Di Virgilio, F., and Steinberg, T. H. (1989). **Phagocytosis**. In Fundamental Immunology, W. E. Paul, ed.: Raven Press Ltd., New York), pp. 703-720.

Songyang, Z., Shoelson, S.E., McGlade, J., Olivier, P., Pawson, T., Bustelo, X.R., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T., Ren, R., Baltimore, D., Ratnofsky, S., Feldman, R.A., Cantley, L.C. (1994). Specific Motifs Recognized by the SH2 Domains of Csk, 3Bp2, Fps Fes, Grb-2, Hcp, Shc, Syk, and Vav. **Molecular and Cellular Biology 14**, 2777-2785.

Straus, D.B, Chan, A.C., Patai, B., and Weiss, A. (1995). SH2 Domain Function is Essential for the Role of the lck Tyrosine Kinase in T Cell Receptor Signal Transduction. **The Journal of Biological Chemistry 271**, 9976-9981.

Straus, D.B., Chan, A.C., Patai, B., and Weiss, A. (1996). SH2 Domain Function is Essential for the Role of the Lck Tyrosine Kinase in T-cell Receptor Signal-transduction. **Journal of Biological Chemistry 271**, 9976-9981.

Straus, D.B., and Weiss, A. (1992). Genetic-evidence for the Involvement of the Lck Tyrosine Kinase in Signal Transduction Through the T-cell Antigen Receptor. **Cell 70**, 585-593.

Susa, M., Rohner, D., and Bichsel, S. (1996). Differences in Binding of PI 3-Kinase to the src-Homology Domains 2 and 3 of p56 lck and p59 fyn Tyrosine Kinases. **Biochemical and Biophysical Research Communications 220**, 729-734.

Swanson, J. A., and Baer, S. C. (1995). Phagocytosis by zippers and triggers. **Trends in Cell Biology 5**, 89-93.

Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H., Yurosaki, T. (1994). Tyrosine Kinases Lyn and Syk Regulate B-cell Receptor-coupled Ca^{2+} Mobilization Through Distinct Pathways. **EMBO Journal 13**, 1341-1349.

Tax, W. J. M., Willems, H. W., Reekers, P. P. M., and et, a. (1983). Polymorphism in mitogenic effect of IgG1 monoclonal antibodies against T3 antigen on human T cells. **Nature 304**, 445-447.

Ting, A. T., Dick, C. J., Schoon, R. A., Karnitz, L. M., Abraham, R. T., and Leibson, P. J. (1995). Interaction between lck and syk family tyrosine kinases in Fcgamma receptor- initiated activation of natural killer cells. **Journal of Biological Chemistry 270**, 16415-16421.

Ting, A.T., Karnitz, L.M., Schoon, R.A., Abraham, R.T., and Leibson, P.J. (1992). Fc-gamma Receptor Activation Induces the Tyrosine Phosphorylation of Both Phospholipase-C (PLC)-gamma-1 and PLC-gamma-2 in Natural-Killer-Cells. **Journal of Experimental Medicine 176**, 1751-1755.

Valerius, N. H., Stendahl, O., Hartwig, J. H., and Stossel, T. P. (1981). Distribution of actin-binding protein and myosin in polymorphonuclear leukocytes during locomotion and phagocytosis. **Cell 24**, 195-202.

Van de Winkel, J. G. J. (1996). Human IgG Fc Receptors (Editor).

Van de Winkel, J. G. J., and Capel, P. J. A. (1993). Human IgG Fc receptor heterogeneity: Molecular aspects and clinical implications. **Immunology Today 14**, 215-221.

Van Den HerikOudijk, I. E., Capel, P. J. A., Van Der Bruggen, T., and Van De Winkel, J. G. J. (1995). Identification of signaling motifs within human FcgammaRIIa and FcgammaRIIb isoforms. **Blood 85**, 2202-2211.

Van Der Geer, P., Hunter, T., and Lindberg, R.A. (1994). Receptor Protein-Tyrosine Kinases and Their Signal-transduction Pathways. **Annual Review of Cell Biology 10,** 251-337.

Van Oers, N.S.C., Killeen, N., and Weiss, A. (1996). Lck Regulates the Tyrosine Phosphorylation of the T cell Receptor Subunits and ZAP-70 in Murine Thymocytes. **Journal of Experimental Medicine 183,** 1053-1062.

Wang, A. V. T., Scholl, P. R., and Geha, R. S. (1994). Physical and functional association of the high affinity immunoglobulin G receptor (FcgammaRI) with the kinases Hck and Lyn. **Journal of Experimental Medicine 180,** 1165-1170.

Warmerdam, P. A. M., Van De Winkel, J. G. J., Gosselin, E. J., and Capel, P. J. A. (1990). Molecular basis for a polymorphism of human Fcgamma receptor II (CD32). **Journal of Experimental Medicine 172,** 19-25.

Weiss, A. (1993). T-cell Antigen Receptor Signal Transduction - A Tale of Tails and Cytoplasmic Protein-Tyrosine Kinases. **Cell 73,** 209-212.

Weissenhorn, W., ECK, M.J., Harrison, S.C., and Wiley, D.C. (1996). Phosphorylated T Cell Receptor ζ-chain and ZAP70 Tandem SH2 Domains Form a 1:3 Complex *in vitro*. **European Journal of Biochemistry 238,** 440-445.

Weissman, A.M., Ross, P., Luong, E.T., Garciamorales, P., Jelachich, M.L., Biddison, W.E., Klausner, R.D., and Samelson, L.E. (1988). Tyrosine Phosphorylation of the Human T-cell Antigen Receptor Zeta-chain - Activation via CD3 but not CD2. **Journal of Immunology 141,** 3532-3536.

Whyte, M. K. B., Hardwick, S. J., Meagher, L. C., Savill, J. S., and Haslett, C. (1993). Transient elevations of cytosolic free calcium retard subsequent apoptosis in neutrophils in vitro. **Journal of Clinical Investigation 92,** 446-455.

Zack, D.J., Stempniak, M., Wong, A.L., and Weisbart, R.H. (1995). Localization of an Fc-Binding Reactivity to the Constant Region of Human IgG4. **The Journal of Immunology 155,** 5057-5063.

Appendix I

Cloning and Phagocytosis Experiments

Maxi and Mini Prep Solutions

GTE (mini-prep)

Glucose	50mM
Tris-HCl pH 8.0	25mM
EDTA	10mM
Lysozyme	4mg/ml

Potassium Acetate Solution (mini-prep)

Glacial Acetic Acid	11.5ml
5M Potassium Acetate	60ml
dH_2O	28.5ml

Maxi-Solution I

EDTA	10mM

Maxi-Solution II

SDS	1% SDS
NaOH	0.2M

Maxi-Solution III

Glacial Acetic Acid	11.5ml
5M Potassium Acetate	60ml
dH_2O	28.5

SOC Medium

Bacto-typtone	5g
Bacto-yeast extract	1.25g
NaCl	0.125g
KCl (250mM)	2.5ml
dH_20	250ml

Autoclave	
2.0M $MgCl_2$(sterile)	1.25ml
1.0M Glucose(sterile)	5ml

TE Buffer

Tris pH 8.0	10mM
EDTA	1mM

TAE

50x stock	
Tris Base	242g
Glacial acetic acid	57.1ml
0.5M EDTA pH 8.0	100ml

Competent Cell Preparative Solutions

Tfb I

KCl	100mM
$MnCl_2$	50mM
KOAc	30mM
$CaCl_2$	10mM
Glycerol	15% v/v
EDTA	0.2mM

Tfb II

$CaCl_2$	75mM
Na-MOPS pH 7.0	10mM
KCl	10mM
Glycerol	15% v/v

Tissue Culture Solutions

Phosphate Buffered Saline

20x stock (autoclaved)	
NaCl	80g
KCl	2g
$Na_2HPO_4 \cdot 2H_2O$	11.5g
KH_2PO_4	2g
dH_2O pH 7.4	up to 500ml

NU Medium

NU serum	
(Collaborative Research)	10%
Glutamine	2mM
Penicillin (Gibco BRL)	100IU/ml
Streptomycin (Gibco BRL)	100µg/ml

Added to a volume of DMEM medium

Immediately prior to use chloroquine is added

Chloroquine	100µM

Elutip-D Solutions

High Salt

NaCl	1M
Tris (pH 7.4)	20mM
EDTA	1mM

Low Salt

NaCl	0.2M
Tris (pH 7.4)	20mM
EDTA	1mM

Trypsin

10x stock

Trypsin (13,000-20,000 U/mg)	50mg
0.5M EDTA	1ml
1xPBS	99ml

RPMI Medium

Fetal Calf Serum	10%
Glutamine	2.0mM
Penicillin (Gibco BRL)	10mg/ml
Streptomycin (Gibco BRL)	10mg/ml

Components were added to a volume of RPMI supplied by Imperial

Hepes RPMI Medium

50mM

10% Calf Serum DMEM Medium

New Born Calf Serum

(Gibco BRL)	10%
Glutamine	2.0mM
Gentamicin	15μg/ml
Penicillin (Gibco BRL)	100IU/ml
Streptomycin (Gibco BRL)	100μg/ml

Components were added to a volume of DMEM (Dulbecco's Modified Eagles Medium) supplied by Imperial

Tetracyclin Preparation

tetracyclin (Sigma)	30mg/ml
ascorbic acid	15mg/ml

LB Agar Plates

Premix (Lennox L Agar) supplied by Gibco BRL, Life Technologies

Premix	32g
dH_20	made up to 1L

autoclaved, appropriate antibiotic added, and poured

Premix Components

Agar	12g
SELECT Peptone 140	10g
NaCl	5g
Yeast Extract	5g

TYM Broth

Bacto-Tryptone	2.0%
Bacto-Yeast	0.5%
NaCl	0.1M
$MgCl_2$	0.01M

Lennox L Broth

Premix supplied by GibcoBRL, Life Technologies

Premix	20g
dH_20	made up to 1L

Premix Components

SELECT Peptone 140	10g
NaCl	5g
Yeast Extract	5g

Myeloperoxidase Stain (12 ml)

Hanks' Balanced Salt Solution	6ml
0.2M Phosphate Buffer pH 6.2	5ml

Immediately prior to use the following components were added

1.25mg/ml 0-dianisidine	1ml
30% H_2O_2	15µl

Appendix II

Western and Probing Experiments

RIPA Lysis Buffer	
Tris	50mM
NaCl	150mM
HCl	5M to pH to 7.4
NP-40	1% final concentration
Sodium Deoxycholate (SDS)	0.25% final concentration
EGTA	1mM
on day of use	
PMSF	1mM
CLAP(chymostatin, leupeptin, antipain, and pepstatin)	1µg/ml
Sodium Orthophosphate	1mM
Sodium Fluoride	1mM

Stacking Gel	
40% Acrylamide (BioRad)	2ml
10% SDS	200µl
0.5M Tris pH 6.8	5ml
dH_2O	12.6ml
10% Ammonium Persulfate(BioRad) (made up 0.1g in 1ml dH_2O)	160µl
TEMED (BioRad)	40µl

Separating gel	
10% Acrylamide	
40% acrylamide (BioRad)	7.5ml
10% SDS	300µl
1.5M Tris pH 8.8	7.5ml
dH_2O	14.4ml
10% Ammonium Persulfate (BioRad)	270µl
TEMED (BioRad)	30µl

Protein Sample Buffer

3x buffer	
0.5M Tris pH 6.8	3.75ml
SDS	0.9g
Glycerol	3ml
Bromo-phenol blue	3µl 0.1%w/v
β-mercapto-ethanol	2µl
dH_20	up to 10ml

SDS-Page Running Buffer

10x stock	
Glycine	144.2g
Tris Base	30.3g
SDS	10g
dH_20	up to 1 liter

Western Blotting Transfer Buffer

Glycine	72g
Tris Base	15g
Methanol	1L
dH_20	up to 5 liters

Tris Buffered Saline (TBS)

1x TBS	
Tris pH 7.5	100mM
NaCl	150mM

TBS - Tween (TBS-T)

Tween 20	0.1%
To volume with TBS	

Blocking solution

Non-fat milk (Marvel; Premier Beverages)	
1x TBS	
To long-term solutions azide was added	
Azide	0.01%

Ponceau Staining Solution

Ponceau S (sodium salt)	0.1% weight per volume
Acetic Acid	5.0%

Griess Solutions

Solution A

Alfa-naphtyl-amine	0.1%
dH_20 to volume	

Solution B

Sulfanilamide	1.0%
Phosphoric acid	5.0%