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Studies of $Fc_{gamma}R$ signalling: I. A chimeric HSA-muFc_{gamma} RIIb2 receptor. II. huFc_{gamma}RIIA: Induced tyrosine phosphorylation

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II. huFc_{gamma}RIIA--Induced Tyrosine Phosphorylation

by

Catherine Jean Painter

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

STUDIES OF $Fc_{gamma}R$ SIGNALLING

I. A CHIMERIC HSA-muFc_{gamma}RIIb2 RECEPTOR

II. huFc_{gamma}RIIA--INDUCED TYROSINE PHOSPHORYLATION

by

Catherine Jean Painter

Advisor: Professor Jay C. Unkeless

Fc receptors for IgG bind IgG via the Fc domain, linking humoral and cellular immunity. Elucidation of signalling pathways has been exciting, with the recent realization that several immune receptors share a common tyrosine activation motif. This motif is necessary and sufficient for T cell receptor signalling.

Study of a chimeric HSA-muFc_{gamma}RIIb2 receptor expressed in the P388D1 cell line revealed that the extracellular domain of the muFc_{gamma}RIIb2 receptor is *not* required for signalling for phagocytosis of immune complexes. Thus, crosslinking of Fc_{gamma}Rs, in the absence of any conformational change in the extracellular domain, is sufficient to initiate signalling.

Activation of huFc_{gamma}RIIA led to rapid tyrosine phosphorylation and dephosphorylation of distinct cellular proteins. Herbimycin A pretreatment demonstrated that the tyrosine phosphorylation response is required for $[Ca^{2+}]_i$ flux and phagocytosis. Severe truncations of the cytoplasmic domain abolished all functioning. The Δ264 receptor, missing 17 carboxyl--terminal amino acids including the downstream YXXL of the motif, is capable of a *partial* tyrosine phosphorylation response. The subset of tyrosine phosphorylation preserved in Δ264

may be involved in phagocytosis of immune complexes mediated by this receptor, but is not sufficient for the $[Ca^{2+}]_i$ flux abrogated by this deletion.

While nine of ten various point mutants were phenotypically wildtype, mutation of the tyrosine of the upstream YXXL dyad to phenylalanine (Y252F) resulted in a significantly crippled receptor, incapable of $[Ca^{2+}]_i$ flux or phagocytosis. The tyrosine phosphorylation response of Y252F, while diminished in intensity, was largely *intact*, with a major exception of a 51,000 Da protein. The absence of tyrosine phosphorylated p51 from the response of both Δ264 and Y252F, which are both incapable of $[Ca^{2+}]_i$ flux, suggests that p51 is an early and important constituent of the signalling pathway of huFc$_{gamma}$RIIA. The phosphorylation response of Y252F was delayed but *dephosphorylation* was significantly delayed, suggesting that protein tyrosine phosphatases involved in the signalling cascade are not constitutively active but are *induced* upon receptor engagement.

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I BACKGROUND AND SIGNIFICANCE

Overview

Fc receptors for IgG, FcγRs, form a family of receptors that specifically bind IgG via the Fc domain and that mediate physiologic functions. These receptors and soluble Ig binding factors play important roles in immunity, which can be grouped into three areas: cellular immune defense and lymphocyte regulation, immunoglobulin transcytosis, and autoimmune pathology. The crosslinking of FcγRs on phagocytic cells activates a variety of leukocyte responses, including phagocytosis, antibody-dependent cell--mediated cytotoxicity (ADCC), and the release of reactive oxygen intermediates, lysosomal hydrolases, arachidonate metabolites, and other mediators of inflammation. In addition, the binding of immune complexes to NK cells and macrophages can also alter the state of activation of the cell, inducing the transcription and synthesis of lymphokines such as TNF-α, IFN-γ, and GM-CSF, and receptors for activating cytokines such as IL-2. The role of the FcγRs present on B cells and a small subset of T cells is not clearly understood, but they may function to blunt the immune response. Soluble immunoglobulin binding factors (IBFs), as well as coaggregation of the antigen receptors (membrane--bound Ig) with FcγRs inhibit B cell differentiation (1,2). A similar finding has recently been reported for Fc$_\epsilon$RII (CD23) (3). Though not yet tested, it seems likely that FcγRs on neonatal rat gut epithelium (4) and syncytiotrophoblasts (5) are involved in transcytosis of immunoglobulin. Dysfunction of macrophage FcγRs (6,7) as well as the presence of high titers of

anti-FcγR immunoglobulin (8,9) have been reported in both human and mouse autoimmune disease.

FcγRs differ from many other receptor classes in that crosslinking or immobilization of the receptors in the membrane by polyvalent immune complexes, rather than binding of ligand *per se* is the triggering event. This is true for both receptors with low affinity (such as FcγRII or CD32) and for receptors with high affinity. The paradigm for the latter class is Fc$_\epsilon$RI, which binds IgE with an affinity $> 10^9$ M^{-1} (see (10) for review). Degranulation of mast cells or basophils does not occur until the crosslinking of Fc$_\epsilon$RI is initiated by the binding of multimeric IgE-antigen complexes. Reagents (such as anti-FcR antibodies) that crosslink the receptor by binding to epitopes other than those involved in the immunoglobulin Fc binding site are effective triggers (10-18). The sufficiency of this nonphysiological crosslinking to induce signalling suggests that binding of ligand by itself does not result in transmission of a conformational change to the cytoplasmic or transmembrane domains. Anti-CD3 and anti-TCR antibodies are also able to induce signalling in various T cell systems (19). There may, however, exist preferred orientations of receptors engaged in signalling as is suggested by reports that indeed some mAbs directed against Fc$_\epsilon$RI are not capable of initiating signalling when used to crosslink the receptor (12).

All FcRs, except CD23 (Fc$_\epsilon$RII), are members of the Ig supergene (20) family and are homologous to each other. Most FcγRs are class I membrane glycoproteins with one transmembrane domain and a cytoplasmic domain. However, huFcγRIIIB is anchored in the neutrophil plasma membrane by a glycan phosphatidyl

inositol (GPI) moiety (21-25). Low avidity forms of membrane bound FcγRs contain two extracellular Ig-like regions, whereas high avidity forms contain three Ig-like regions. Assigning functions to individual FcγRs has been a challenging task since within a subclass receptors share immunologically indistinguishable extracellular domains and their cellular distributions overlap considerably. This section will summarize the current state of knowledge of the structure, function, and signalling mechanisms of mouse and human FcγRs. Elucidation of the signalling pathways has been an especially exciting area with the recent realization that several varied receptors of the immune system share a common motif (26,27), and this motif has been shown in the T cell receptor (TCR) system to be necessary and sufficient for signal transduction (28-30). Several reviews cover older literature and other aspects of FcγRs, such as autoimmunity, in greater depth (12,16,18,31-35).

Nomenclature

A nomenclature for the family of Fc receptors was agreed upon in June, 1987. However, since then, numerous new FcγR genes have been isolated. The nomenclature that I have utilized is summarized in Table 1, and is in concert with that originally agreed upon while incorporating additional information (12). The species of origin is designated by two lower case letters (eg. mo for mouse and hu for human). The subscript Greek letter refers to the major class of immunoglobulin bound by the receptor. (Thus, FcγRs bind IgG.) The Roman numeral refers to the distinct subclass of the designated receptor class. The subclasses are based on structural similarity and reactivity with specific mAbs. Following the Roman numeral, an upper case letter, A, B, C,..., indicates a distinct gene within that

Name	CD, Ly	Other Names	Affinity	Specificity huIgG	Specificity muIgG	Cellular Distribution MØ	Mono	PMN	NK	B	T	Mast	Baso	Subunit Associations
huFcγRI (a,b1,b2,c)	CD64	-	high (10^8 M^{-1})	1=3>4≫2	2a=3≫1,2b	+	+	+^{i}	-	-	-	-	-	$\alpha\gamma_2$
muFcγRI	-	-	high (>10^7 M^{-1})	3>1>4≫2	2a,2b≫1,3	+	?	?	-	-	-	-	-	?
huFcγRIIA (a1,a2)	CDw32	FcγRIIa	low (10^5 M^{-1})	1=3≫2,4	1≫2a,2b,3	+	+	+	-	-	+	?	?	?
huFcγRIIB (b1,b2,b3)	"	FcγRIIb FcγRIIC	"	"	"	+	+	-	-	+	-	?	?	?
huFcγRIIC	"	FcγRIIa' FcγRIIC	"	"	"	+	+	+	-	-	-^{a}	?	?	?
muFcγRII (b1,b2)	Ly17	FcγRIIβ	low (10^6 M^{-1})	3>1>4≫2	1>2a=2b≫3	+	+	+	-	+	+	+	?	?
huFcγRIIIAα	CD16	FcγRIII-2	medium (>10^6 M^{-1})	1,3≫2,4	3>2a>2b≫1	+	+^{i}	-	+	-	+^{b}	?	?	$\alpha\gamma_2$,$\alpha\zeta_2$, $\alpha\gamma\zeta$
huFcγRIIIB	"	FcγRIII-1	"	"	"	-	-	+	-	-	-	?	?	-
muFcγRIIIα	Ly17	FcγRIIα FcγRIIA	low (10^6 M^{-1})	as for muFcγRII		+^{i}	?	+	+	-	-	+	?	$\alpha\gamma_2$,$\alpha\gamma\zeta$

Table 1 Summary of $Fc_\gamma R$ nomenclature, IgG binding, cellular expression and associated subunits. *a*, negative in T cell lines (Molt-4, Jurkat, Fro-2), peripheral T cells not tested; *b*, only in T_γ subset and CD16/CD3$^+$ lymphocytes; *i*, inducible.

subclass. A specific splice form of a gene is designated with both a lower case letter referring to the gene and Arabic numeral designations. Hence, the alternate splice forms of huFcγRIIB are b1,b2, and b3. The CD nomenclature is also noted for each receptor subclass.

HuFcγRI

Monocytes and macrophages have high affinity (10^8-10^9 M^{-1}) binding sites for human IgG1 and IgG3 and for murine IgG2a and IgG3 (36,37). FcγRI is univalent for human IgG1 (38). Several anti-huFcγRI mAbs have been described, including mAb 32 (39), mAbs 22, 44, and 197 (40), and mAb 10.1 (41). None of these, as Fab fragments, will inhibit the binding of monomeric IgG. The purified receptor has a M_r, as determined by SDS-PAGE, of 72,000 (42,43), which decreases after removal of N-linked carbohydrate to 40,000-50,000 (44,45).

Very recent work has identified three highly homologous genes (A, B, and C) which encode for four huFcγRI transcripts, a1, b1, b2, and c1 (46). The A gene product, which was originally cloned using a eukaryotic expression shuttle vector (47), encodes a transmembrane protein containing six potential N-linked glycosylation sites and six cysteine residues, which are presumably disulfide linked to form three C2-set Ig-like domains (20). In contrast, huFcγRII and huFcγRIII encode only two Ig-like domains. The B gene gives rise to two transcripts, one encoding a transmembrane protein identical to that of the a1 transcript, but lacking the third membrane--proximal extracellular domain that is believed to endow high affinity binding of IgG (48). Another transcript of the B gene, as well as the c1 transcript encode secreted forms of the receptor that lack any transmembrane and

cytoplasmic domains (46). The transmembrane domain of the a1 product, which appears to be the receptor previously identified with various antibodies (46), is 21 residues and the cytoplasmic domain is short and highly charged (47). The mouse FcγRI is similar in structure (49). Homology also exists between the first two N-terminal external Ig-like regions of each FcγRI and the analogous domains of mouse and human FcγRII and huFcγRIII (47,49).

HuFcγRI has been shown to associate with the homodimeric actin--binding protein (ABP) (50) *in vitro* when the receptor is not occupied by monomeric IgG. The authors hypothesized that this result might apply *in vivo* to the ventral surface of spread macrophages (*e.g.* adherent to the sinusoidal endothelial cells of liver, spleen, or lymph nodes), with minimal or no exposure to *ambient* IgG molecules (51,52). They further hypothesized that macrophage adherence might be stabilized by a continuous lattice established between FcγRI, ABP, and the submembranous actin cytoskeleton (53). The likelihood of IgG exclusion from the ventral surface is questionable, however. The original study on which the authors based their hypothesis was limited to *in vitro* study of the spreading of macrophages onto IgG coated surfaces, in the absence of soluble IgG. In this special case, a "sealing" was observed between the ventral surface and the surrounding medium (51). It is also unclear, when considering this hypothesis, what the authors would propose happens to the monomeric IgG with which the huFcγRI is normally saturated. Perhaps a monomeric IgG free state of huFcγRI could be envisioned on the ventral surface of long term resident and immobile macrophages. A more plausible explanation put forth by the authors is that as their studies were carried out under nonequilibrium

conditions, the results do not rule out a lower *in vivo* affinity of interaction between huFcγRI and ABP. Thus, huFcγRI which is normally saturated with monomeric IgG under most *in vivo* conditions, may interact with ABP whereas aggregation of the receptor may serve to disrupt the ABP interaction, resulting in increased membrane fluidity at the region of receptor stimulation (50).

Very recent work has identified yet another intermolecular association with the γ subunit. The γ subunit, whose role in FcR signalling will be further discussed below, is a disulfide--linked homodimer between two chains of 7000 M_r. The γ homodimer is also present in the FcγRIIIA and the Fc$_\epsilon$RI receptor complexes, and both these receptors share a nearly identical stretch of ten amino acids, including a negatively charged aspartyl residue, in their transmembrane domains: $V^I/_L$LFAVDTGL (referred to herein as the γ transmembrane sequence). This unusual sequence has been implicated in mediating the association of these receptors with the γ subunit (54,55). A corresponding region in the transmembrane domain of the γ subunit mediates receptor complex assembly and prevents degradation of the ligand-binding α-chain of the receptor in the endoplasmic reticulum (56). A sequence present in the transmembrane domain of huFcγRI shares identity with the γ transmembrane sequence at four-five of the ten residues and has conservative replacements in the others: GIMFLVNTVL. The most notable difference is the substitution of a positively charged asparagine for negatively charged aspartate residue. It has been shown that mutating the aspartate to valine in the transmembrane domain of huFcγRIIIAα lessened the dependence on γ chain for receptor expression (57). This difference may explain why huFcγRI is not

dependent on coexpression of the γ chain for surface expression (46) nor indeed for phagocytic function [unpublished observations in (46,58,59)]. The role of the γ subunit in FcγRI signalling and function will be an exciting area of current study, and will be guided by the results already obtained in the T cell receptor and FcγRIIA and Fc$_\epsilon$RI systems, which will be discussed below.

Members of the Ig-supergene family are characterized by a variety of heterotypic interactions that are central to cell-cell recognition. The extracellular Ig-like domains interact with and bind the Fc domain of IgG molecules. Structural features of the IgG Fc region that contribute to huFcγRI high affinity binding of murine IgG2a and human IgG1 have been delineated with the greatest precision. Comparison of amino acid sequences of IgG subclasses from different species that bind to huFcγRI with high affinity revealed the highly conserved sequence Leu^{234}-Leu-Gly-Gly-Pro in the hinge portions of the C_H2 domain (60). The sequence of murine IgG2b, a low affinity ligand was identical in this region except for Glu^{235}. Site-directed mutagenesis to convert IgG2b Glu^{235} $\rightarrow$ leu converted a low affinity ligand to a high affinity ligand comparable to murine IgG2a and human IgG1 (61).

In addition, out of a panel of mAbs directed against epitopes in the C_H1, C_H2, C_H2-C_H3 junction, and C_H3 domains, only those mAbs directed against the N-terminal region of C_H2 inhibited binding of ^{125}I-labeled IgG to monocytes and U937 cells. Once IgG was bound to huFcγRI, the hinge region-specific mAbs could not bind. The C_H2-C_H3 interdomain region mAbs inhibited binding more weakly. Monocyte ADCC could be inhibited using the same C_H2-domain specific mAbs (62).

Binding of IgG to huFcγRI requires only one C_H2 domain. Hybrid IgG1-2a, and IgG2a-2b mAbs produced by hetero-hybridomas bind to huFcγRI with normal K_a to the same number of sites and compete with native murine IgG2a or human IgG1 (63). Since neither murine IgG1 or IgG2b bind to huFcγRI, the high affinity binding has to be mediated by the single IgG2a H chain in the heterodimeric IgG.

All normal IgG has N-linked oligosaccharide in the C_H2 domain and the locus of the glycosylation site is conserved. Glycosylation is important for the binding of IgG to huFcγR. Murine IgG2a produced by hybridomas grown in the presence of tunicamycin binds C1q and activates C1 nearly normally, but has a greater than fifty-fold reduction in the K_a for binding to monocyte huFcγRI (64). That minimal changes seem to occur in C1q binding and the activation of C1 indicates that there is no gross structural change in the C_H2 domain. Furthermore, isolated oligosaccharides do not inhibit binding of IgG to monocytes which strongly suggests that they are not directly involved in huFcγRI-IgG binding (60). Thus the carbohydrate in the C_H2 domain of the Fc region probably serves to maintain tertiary structure by forcing the two H chains apart.

The huFcγRI binding site contains a readily oxidizable residue. Porphyrin photosensitization *in vitro* of monocytes and U937 cells selectively reduced murine IgG2a binding to huFcγRI (65). This was not due to loss of the receptor from the cell surface, as anti-huFcγRI mAbs, still bound to the cells. Scavenger experiments suggest that generation of superoxide radical is responsible for the reduced binding. Thus, during the superoxide burst triggered by chemotactic peptides, or in the lungs

of smokers, there may be a significant inactivation of FcγRI activity on mononuclear cells.

It is not clear why there are multiple FcγRs, as many of the functions are subtended by more than one receptor. Indeed, several members of a Belgian family have a complete absence of huFcγRI expression on their peripheral blood monocytes (66-68). Two explanations of their apparent good health are the following: 1) These individuals may possess a developmental defect such that huFcγRI is not expressed on monocytes but is expressed on tissue macrophages where it functions normally; such a somatic cell defect is considered unlikely, however. 2) An absence of huFcγRI may be of little consequence due to the redundancy of functions among leukocyte FcγR (14,69).

HuFcγRII

A second subclass of human FcγR, huFcγRII (CD32), was initially identified by affinity chromatography of U937 lysates on IgG-Sepharose (42). The anti-huFcγRII mAb IV.3 (70) immunoprecipitates an antigen of about 40 kDa. HuFcγRII is found on monocytes, neutrophils, platelets, B cells, eosinophils (71,72), basophils (73) and trophoblasts (5). The receptor binds aggregated IgG with low avidity (Ka= 1-3 x 10^6 M^{-1}). Monomeric IgG binding cannot be demonstrated unless media of low ionic strength is used (74). The affinity with which huFcγRII binds IgG subclasses is the following: $IgG_1 = IgG_3 > > IgG_2 = IgG_4$ (75-77).

The observation that mAb IV.3 did not react with Daudi cells, although a 40 kDa huFcγR could be immunoprecipitated with a polyclonal anti-huFcγRII serum (78), suggested the possibility of isotypic variation. MAb IV.3 reacts with the 40

kDa receptor on neutrophils, macrophages, and platelets and mAbs 41H.16, KuFc79, and KB61 recognize another 40 kDa molecule on B cells, neutrophils, and macrophages (79,80). Recent studies have shown that some crossreactivity for huFcγRII forms is evident among these and other huFcγRII-specific mAbs (81,82). The mAb IV.3 either does not stain B cells (Dr. Peter Boros, unpublished communication) or stains B cells very weakly (82). Initial cDNA clones of huFcγRII appeared to be nearly identical products of a single gene with no differential splicing (83,84). Subsequently, additional cDNA clones were isolated showing that at least three genes encode huFcγRII proteins (5,85-87). All of the huFcγRIIs have homologous extracytoplasmic domains and are most homologous to muFcγRII, especially huFcγRIIB. The nomenclature is summarized in Table 1. HuFcγRIIA [originally referred to as huFcγRIIa in ref. (86)], huFcγRIIB [called huFcγRIIb in ref. (86) and huFcγRIIC in ref. (84)], and huFcγRIIC [called huFcγRIIa' in ref. (86) and huFcγRIIB in ref. (84)] are the currently accepted names for the three members of this subclass.

Extensive sequence homology exists among the various huFcγRII mRNA molecules in the encoded extracellular and transmembrane domains as well as in the 3' untranslated sequences. FcγRIIB sequences differ from FcγRIIA only in the signal sequence and in part of the cytoplasmic domain while IIB differs from FcγRIIC only in the cytoplasmic domain. The FcγRIIA gene gives rise to two transcripts differing only in the polyadenylation site, and FcγRIIC encodes only a single transcript. FcγRIIA and FcγRIIC are distinguished only by their signal sequences: that of FcγRIIA is homologous to the murine FcγRIII signal sequence,

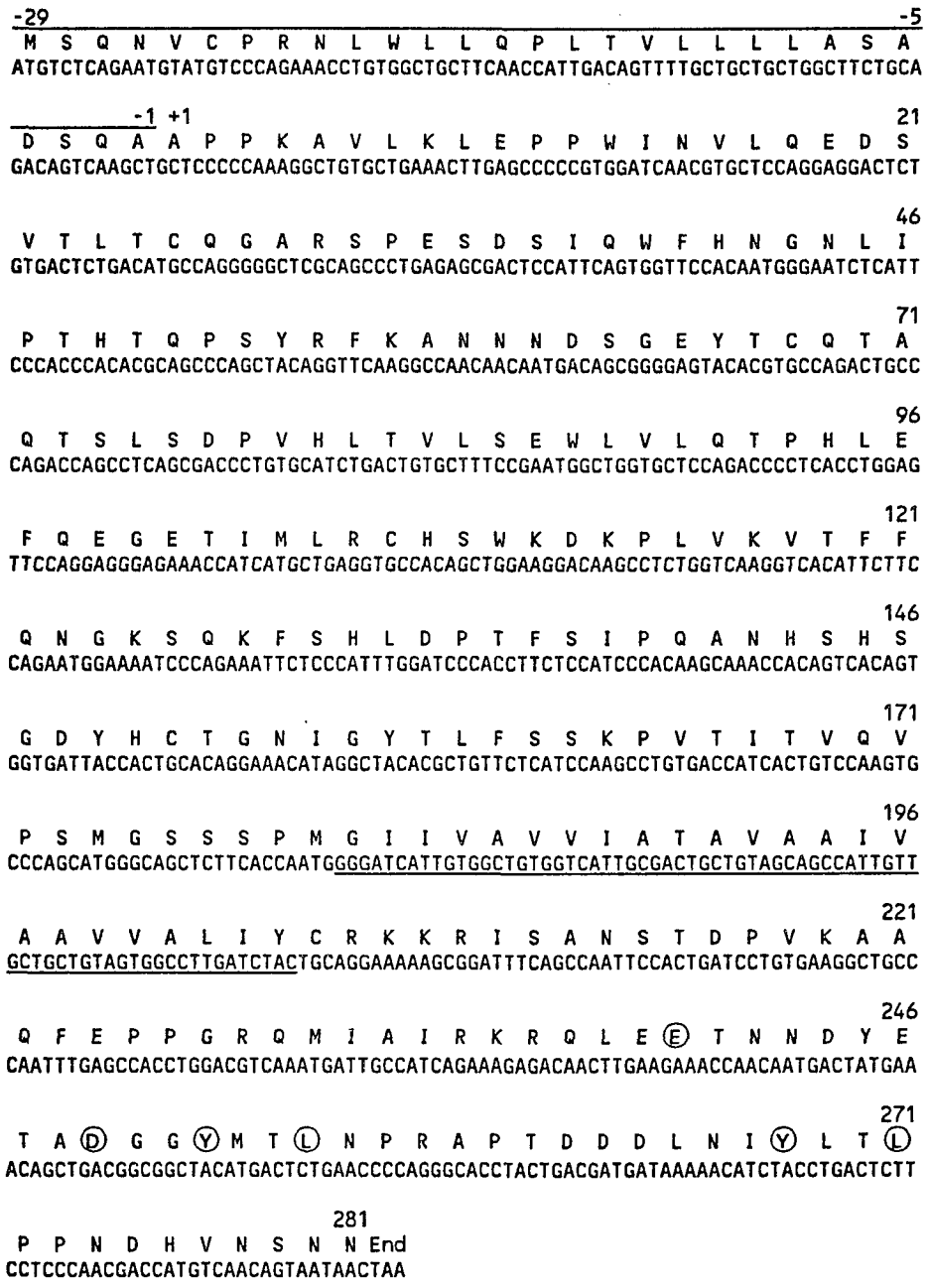


Figure 1 HuFcγRIIA sequence. The putative signal sequence is overlined, and the transmembrane sequence is underlined. The codon encoding the first amino acid of the mature protein is numbered +1. The residues of the conserved MIRR motif (see refs. 26,27) are encircled.

while the signal sequence of FcγRIIC is homologous to that of murine FcγRII. Otherwise, FcγRIIA and FcγRIIC are nearly identical. The cytoplasmic domain of IIB, which differs from FcγRIIA and FcγRIIC, is most homologous to that of muFcγRII. Indeed, both the human and murine FcγRII genes undergo differential splicing in their cytoplasmic domains (12,86,88).

The cellular distribution of each huFcγRII mRNA transcript was analyzed. HuFcγRIIA and FcγRIIC are expressed in neutrophils, cultured adherent monocytes, chronic myelogenous leukemia cells, various monocyte-like cell lines, DMSO-differentiated HL-60, and the erythroleukemic cell line K562; FcγRIIA and FcγRIIC are not expressed in lymphocytes or NK cells (12,86). Of five lymphoid cell lines (Daudi, Raji, AW RAMOS, IM-9, and MOLT-4), only the Burkitt lymphoma cell line Daudi expresses huFcγRIIA. The FcγRIIA and FcγRIIC genes are distinguished by their signal sequences, but give rise to essentially the same final protein products, albeit with several amino acid substitutions. The cellular distribution of huFcγRIIB includes monocytes, macrophages, lymphocytes, and various pluripotential cell lines (12,86); expression of IIB has not been detected in NK cells, neutrophils, nor T cell lines (12). Both huFcγRIIB and muFcγRII undergo differential splicing in their cytoplasmic domains (86,88). The splice-form of huFcγRIIB detected in each cell type was not determined. In summary, huFcγRIIB is preferentially expressed in lymphocytes, while FcγRIIA and FcγRIIC are preferentially expressed in neutrophils; monocytes express all three classes (12). *In situ* hybridization studies showed that huFcγRIIB is present in syncytiotrophoblast cells of placenta (5).

Monoclonal Abs are not available that will discriminate rigorously between the three major isoforms of hu FcγRII, owing to the great homology of their extracellular domains. However as noted above, the widely used mAb IV.3 either does not stain B cells (Dr. Peter Boros, unpublished communication) or stains B cells very weakly (82). In addition to the isotypic variation, there are also FcγRIIA allotypes HR (high responder) and LR (low responder) which differ in their ability to bind murine IgG1. The HR allotype that binds murine IgG1 has Arg_{133} substituted for His_{133} (89,90), and can be distinguished by a slightly different isoelectric focusing pattern (91).

There are differences in the sensitivity of FcγR to proteases that may affect their function. In the case of huFcγRII, the binding of immune complexes and erythrocytes opsonized with murine IgG1 to monocytes or K562 cells (which express only FcγRIIA) is increased following treatment with trypsin or pronase (92). The avidity of binding is also increased by incubation of monocytes or K562 cells with neutrophil elastase or with supernatants of cells stimulated with fMLP (93). Of interest is the observation that the FcγRII expressed on human B cell lines did not show any alteration in M_r following protease treatment. Monocyte binding of murine IgG1-sensitized E was inhibited by both macromolecular inhibitors of serine proteases and by low molecular weight inhibitors such as diisopropyl fluorophosphate and tosyl-L-lysyl chloromethyl ketone (94). Thus there may be an *obligatory* proteolytic event required for binding by FcγRII. However, this event may not involve the FcγRII molecule itself, since the number of receptors, and the mobility of the FcγRII are unchanged by proteolysis (93,94).

The generation of superoxide itself may result in activation of FcγRII for phagocytosis (95,96). Priming by GM-CSF or TNF-α induces a twofold increase in phagocytic index of EIgG by neutrophils, which is inhibited by the addition of superoxide dismutase. The increase in phagocytosis attributable to the production of superoxide is not seen in neutrophils from patients with chronic granulomatous disease, in which the respiratory burst is absent (95). These results are reminiscent of the protease activation of FcγRII discussed above. The effects of proteases and oxidation may be due to subtle changes in the receptor, or to changes in other plasma membrane proteins.

HuFcγRIII

HuFcγRIII (CD16), binds IgG1 and IgG3 with an intermediate affinity (K_a of $\sim 4 \times 10^6\ M^{-1}$) (97) and is expressed on macrophages, NK cells, neutrophils, eosinophils, and some T cells (32). HuFcγRIII on SDS-PAGE runs as a broad smear between 50,000 and 70,000 M_r, probably due to glycosylation heterogeneity (98). Immunoprecipitation studies of NK and neutrophil cell lysates using a huFcγRIII-specific mAb followed by deglycosylation and SDS-PAGE revealed core proteins of different M_r in the two cell types (99). Subsequent cDNA cloning experiments demonstrated that the NK cell transcribes an mRNA distinct from that of neutrophils (23-25,100,101). In fact, two genes with cell type specific transcription encode huFcγRIIIs: huFcγRIIIA, expressed in NK cells and macrophages, and huFcγRIIIB, which is expressed in neutrophils.

Initial attempts to express huFcγRIIIA, as well as the homologous muFcγRIII in fibroblast lines were unsuccessful. These two receptors have an unusual eight

amino acid stretch which includes a charged aspartyl residue in their transmembrane domain which is identical to that of the ligand--binding α subunit of the rat $Fc_\epsilon RI$. $Fc_\epsilon RI$ is a multisubunit structure composed of α, β, and γ-homodimer polypeptides ($\alpha\beta\gamma_2$). The γ chain is required for membrane expression of the rodent and human $Fc_\epsilon RI$ complex; the β subunit is also required in the human receptor (12,102-108). NK cells that only express $Fc\gamma RIII$ contain γ and ζ transcripts. Cotransfection of $Fc\gamma RIII$ cDNA and γcDNA resulted in 50-100 fold increase in surface expression in COS-7 cells and physical association of $Fc\gamma RIII$ with γ was demonstrated in a murine macrophage line (109). In NK cells, $huFc\gamma RIIIA$ associates predominantly perhaps with γ_2, but also with ζ_2, ζ-γ, and γ-η dimers (110-115). These three subunits, γ, η, and ζ, are members of the newly defined zeta gene family (105,115,116). Comparison of conserved residues of these subunits and mutational analysis have shown that the transmembrane domain of γ, including a critical leucine residue, is required for subunit interaction with $Fc\gamma RIIIA\alpha$. Moreover, this interaction masks an epitope on the α chain which otherwise encodes for rapid degradation of the subunit in the ER and allows the $\alpha\gamma_2$ receptor to be transported to the Golgi (56).

The ζ subunit was originally recognized as a component of the T cell receptor complex (116). Alternate splicing in the cytoplasmic domain of the ζ gene gives rise to the η protein. The γ and ζ genes are highly homologous in sequence and organization, reflecting a gene duplication event (105). Various homodimers and heterodimers of the zeta family are associated with the TCR, $Fc_\epsilon RI$, and $Fc\gamma RIII$ receptors(112,114) and are involved in the signal transduction pathways of these

receptors (30,110,111,114,117-123) as will be further discussed below. Each of these receptors, as well as the B cell antigen receptor (BCR), are multichain complexes that signal when crosslinked by the appropriate multivalent ligand. They have been termed *multichain immune recognition receptors* (MIRR) by Keegan and Paul (27). One or more subunits of each MIRR contains a conserved motif in the cytoplasmic domain: D/E-X_7-D/E-X_2-Y-X_2-L-X_7-X_2-Y-X_2-L. The TCR (19) contains α and β antigen binding clonotypic chains, three CD3 chains (CD3-γ, δ, and ϵ), in addition to a zeta family dimer. The MIRR motif is found in two CD3 chains (γ and δ) and in two zeta family chains (γ and ζ). (Please note that CD3-γ is a distinct molecule form the zeta family γ, which is often referred to as $Fc_\epsilon RI\gamma$ or $Fc\gamma RIII\gamma$.) In fact, the ζ subunit has three copies of the motif. The η chain lacks the first half of the motif. The motif is present in both the β and γ subunits of the $Fc_\epsilon RI$, and γ is associated with huFcγRIIIA and muFcγRIII. Recent reports also document association and functional involvement of the β subunit with huFcγRIIIA $\alpha\gamma_2$ complex when all three chains were cotransfected (124,125). The BCR (126,127) is composed of the heavy and light chains of the membrane bound immunoglobulin (Mig), which bind antigen and are clonotypic, as well as two additional subunits, Ig-α and Ig-β (products of the *MB-1* and *B29* genes). Both Ig-α and Ig-β cytoplasmic domains contain the MIRR motif (27). While other FcγR have not been shown to have associated subunits, a variant of the MIRR motif differing principally in the spacing between the two Y-X_2-L units (E-X_8-D-X_2-Y-X_2-L-X_{12}-Y-X_2-L) is found in the cytoplasmic domain of huFcγRIIA and huFcγRIIC (128). As will be further discussed below, the expression of a polypeptide containing a single copy of the

Multichain immune recognition receptors

T cell antigen receptor

⇒7 peptides

α and β (or γ and δ) TcR
CD3 γ, δ, and ϵ
one zeta family dimer: ζ-ζ, ζ-γ, or ζ-η

B cell antigen receptor

⇒ 4 peptides

sIg H_2L_2 tetramer (sIgM or sIgD)
Igα (mB-1)
Igβ (B29)

High affinity IgE receptor (FcϵRI)

⇒ 4 peptides
(mast cells, basophils)

FcϵRI α chain
β chain
γ-γ chain dimer (or ζ-ζ ?)

Moderate Affinity IgG receptor (FcγRIIIA)

⇒1,3, or 4 peptides
(NK cells, MØ, and mast cells)

FcγRIIIA α chain
one dimer: γ-γ, ζ-ζ, or γ-ζ
β chain (mast cells)

High Affinity IgG Receptor (FcγRI)

⇒1 or 3 chains
(monocytes, MØ, induced PMN)

FcγRI α chain
γ-γ chain (monocytes)

Figure 2 Subunit composition of the MIRRs.

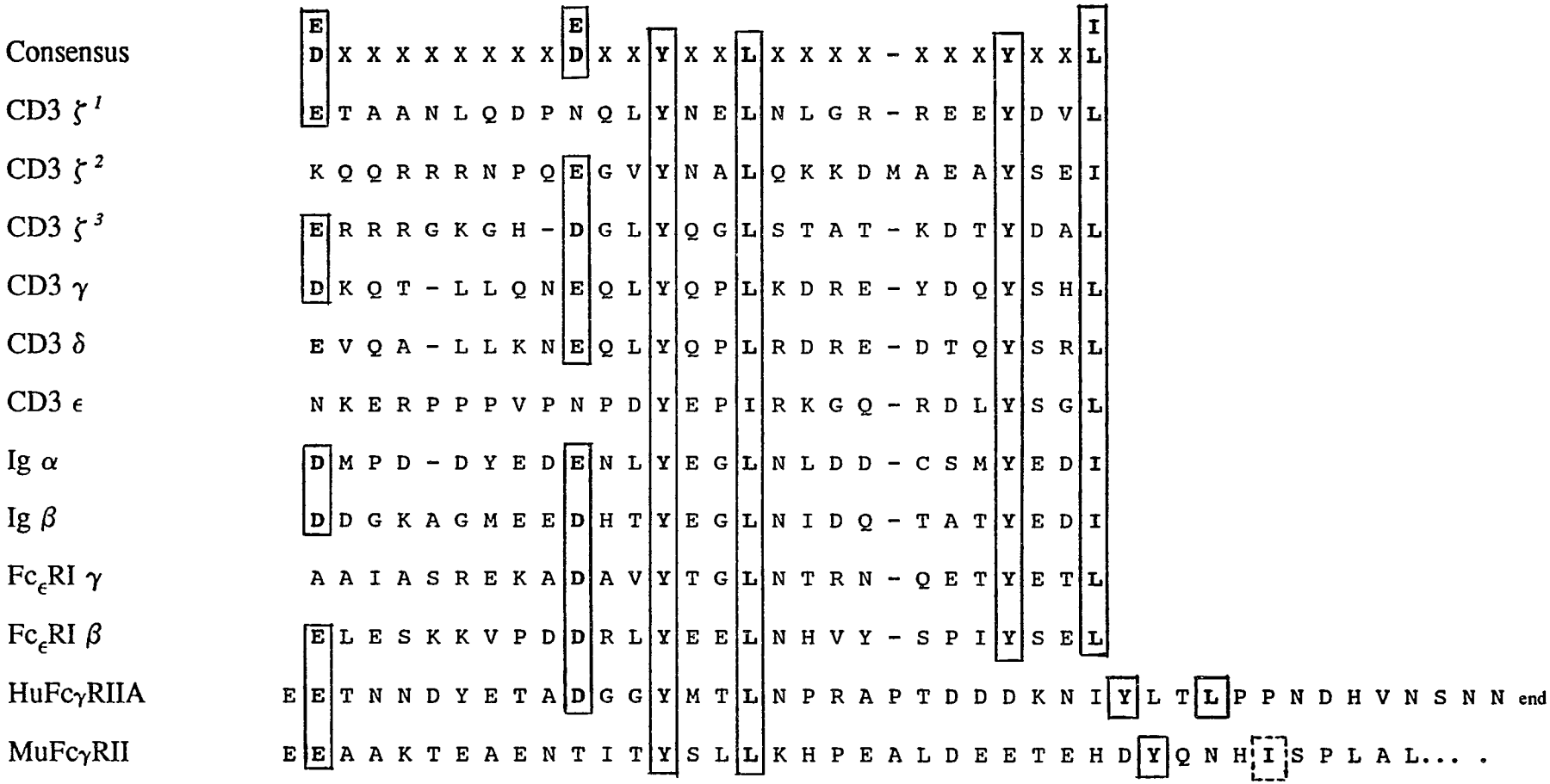


Figure 3 Alignment of the tyrosine activation motif in various MIRRs. All sequences are murine, unless otherwise noted. *1,2,3* -- motif contained in ζ exons 3-4, 4-5-6, and 6-7-8 respectively.

motif is sufficient for many aspects of MIRR signalling. Thus, huFcγRIIA and huFcγRIIC may function fully without the requirement of other subunits. The evolution of multisubunit signalling complexes with duplication of signalling units may be particularly suited for antigen receptors whose ligands are innumerably diverse, resulting in similarly diverse patterns of receptor orientation upon antigen--mediated receptor crosslinking (27). The structure-function relationships and evolutionary implications of this remarkable conservation of both motif and subunit structure among complex and functionally related receptors is elegantly addressed by Keegan and Paul (27).

HuFcγRIIIB, unlike all other FcγRs, is anchored to the neutrophil cell membrane via a GPI linkage and can be released from the cell membrane by a phosphoinositol-specific phospholipase C (23-25,100,101). There is no homologous GPI-anchored FcγR in the mouse. A variable proportion of GPI-anchored proteins are PIPLC-resistant, due to palmitoylation of the inositol ring (129,130), but these molecules remain sensitive to cleavage by an anchor--specific phospholipase D. Neutrophils from patients with paroxysmal nocturnal hemoglobinuria (PNH), a clonal hematopoietic stem cell disorder in which the hematopoietic cells fail to synthesize GPI-anchored proteins, are largely deficient in FcγRIII (25,101). There is, however, some evidence that the loss of all GPI-anchored proteins in PNH is not equal (131,132), and that FcγRIIIB expression is maintained at low levels in the total absence of the GPI-anchored protein DAF (CD55) and CD59 (132). Alteration of single amino acid in the GPI linkage domain of huFcγRIIIB results in anchorage of the protein by a transmembrane domain with a short cytoplasmic tail (57,133).

FcγRIIIA, a type I transmembrane protein, encodes Phe^{203} in the extracellular domain. Site--directed mutagenesis to Ser^{203}, the residue found at the homologous position in FcγRIIIB, results in the synthesis of a GPI-anchored receptor (54,57,133). FcγRIIIB also has a stop codon instead of Arg^{234}, encoding a cytoplasmic domain which, prior to cleavage during GPI-addition, is 21 residues shorter than FcγRIIIA. FcγRIIIA requires a γ subunit for efficient expression at the cell plasma membrane (133). If the FcγRIIIB Ser^{203} is mutated to phe, and the $stop^{234}$ is mutated to Arg, the molecule is expressed at the cell surface only in the presence of γ chain, or the related ζ chain.

Stimulation of neutrophils with chemotactic peptide formyl-met-leu-phe resulted in release of huFcγRIIIB from the neutrophil membrane (22). The mechanism was shown to be proteolytic cleavage, as opposed to the activation of a phospholipase C (134). There are however, differences in the sensitivity of FcγR to proteases that may alter their function, as was discussed above for huFcγRII. Neutrophil FcγRIIIB is sensitive to digestion with leukocyte elastase (135) and pronase (136), suggesting that in a site of inflammation, function of FcγRIIIB may be largely ablated.

As with huFcγRII, two allotypes (NA1 and NA2) exist for huFcγRIIIB. These allotypic differences can cause autoimmune neutropenia in infants (137). Two receptor forms (M_r 19,000 and 21,000) on neutrophils were distinguished after deglycosylation followed by SDS-PAGE (100). The pattern of expression of the 19 kDa and 21 kDa receptor types correlated with the pattern of NA1 and NA2 allotypic marker expression. Discrimination between these allotypes (NA1 and

NA2) was possible using mAbs CLB GRAN11 and GRM1, respectively (138). FcγRIIIA, which is not polymorphic, always types as NA2.

A substantial percentage (25%) of neutrophils in HIV-infected men were negative for huFcγRIIIB expression, while levels of other GPI-linked proteins and huFcγRII were normal (139). The huFcγRIII-negative subpopulation was larger in AIDS diagnosed patients and HIV-infected intravenous drug abusers as compared to HIV-infected homosexuals and uninfected control males. The mechanism responsible for huFcγRIIIB loss and the physiologic consequences of this altered expression have not yet been examined. The levels of huFcγRIII in serum are reported to vary during the course of AIDS, with an initial rise and a subsequent fall in serum huFcγRIII levels in the terminal stages of the disease (139).

MuFcγRI

One of the earliest studies demonstrating the presence of an Fc receptor was the binding by mouse macrophages of erythrocytes sensitized with murine IgG but not with IgM (140). Later studies demonstrated that murine macrophages in fact possess more than one type of FcγR, one of which binds murine IgG2a with high affinity, and others with lower affinity for IgG1, IgG2a, and IgG2b (141-144). A study utilizing *monomeric* IgG2a to determine the kinetic parameters of the muFcγRI found that the Ka varied from 2.2×10^{-7} at 37° to 1.1×10^{-8} at 4°, indicating that the interaction is exothermic (145). As in the case of huFcγRI, the muFcγRI present on peripheral blood cells is fully saturated with circulating monomeric IgG2a. In addition, the pseudo-first order association constant (0.44 min^{-1}) and first order dissociation constant (0.26 min^{-1}), yield a half time of dissociation at 37° of 2.6 min.

Thus, muFcγRI is in rapid equilibrium with IgG2a in the local environment (145). The number of high affinity IgG2a binding sites measured at 4° is 84,000 on P388D1 cells, 110,000 on normal peritoneal macrophages, and 440,000 on thioglycollate--stimulated macrophages. The muFcγRI is trypsin--sensitive (143,145).

MuFcγRI has been cloned (49), and is closely related to huFcγRI (47). (See previous discussion of huFcγRI structure.) The 23 amino acid transmembrane domain is followed by the carboxyl--terminal 84 amino acid cytoplasmic tail. The muFcγRI, when expressed transiently in COS cells, had a Ka of $\sim 5x10^7$ M^{-1} at 0° for IgG2a (49), which is consistent with that obtained the receptor on macrophages (145). Southern analysis suggests that the receptor is encoded by only one gene (49).

MuFcγRII

Two subclasses of low affinity FcγRs are now known to exist in the mouse, muFcγRII and muFcγRIII. Original studies of these receptors failed to discriminate between them as they are 95% homologous in their extracellular domains, and both are recognized by the mAb 2.4G2. Initial work examining protease sensitivity (143,146-148), and ligand binding (141,142,144) as well as the isolation of FcγRII/III loss variants (143) pointed to the presence of multiple murine FcγRs in addition to the high affinity muFcγRI. The development of the first mAb against FcγRII/III, 2.4G2, facilitated studies of its isotype specificity and cellular distribution. MAb 2.4G2 is directed against an epitope associated with the ligand binding site (149) and binds with a Ka of 9.6×10^8 M^{-1} (150), and is now known to recognize both muFcγRII and III. MuFcγRII/III were purified by affinity

chromatography on 2.4G2 sepharose and yielded two major bands of ~60,000 and ~47,000 M_r as visualized on SDS-PAGE (151). The smaller band was likely the muFcγRIII gene product, whose M_r was recently determined (152). The isolated muFcγRII/III, shown to be a sialoglycoprotein, demonstrated a broad ligand isotype specificity--binding complexes of either IgG1, IgG2b, and IgG2a (151). Various expression studies of the cloned cDNAs for muFcγRII have confirmed that the ligand specificity of the recombinant truncated receptor (153), as well as of that expressed on the cell surface (154,155), includes all murine isotypes except IgG3. The relative ligand binding affinity of a recombinant truncated (secreted) form of muFcγRII is IgG1 > IgG2b = IgG2a (153). The mAb 2.4G2 purification results agree closely with other work in which FcγR with ligand binding activity was isolated (156,157).

The cDNAs encoding muFcγRII (originally called muFcγRIIβ) and muFcγRIII (first called muFcγRα) were cloned by three groups (88,154,158). The proteins encoded are both type I integral membrane glycoproteins and are members of the immunoglobulin gene superfamily. The *Ly-17* alloantigen system defines two polymorphisms of the muFcγRII. The adjacent pairs of cysteines in the extracellular domains of murine FcγRII and FcγRIII are each disulfide linked (153,159) and each Ig-like domain has two sites of N-linked glycosylation (159). The two proteins are 95% homologous in the extracellular domains (of 185 or 181 amino acids) due to an identical stretch of 171 amino acids (88). Neither the transmembrane domains (of 20 or 26 residues) nor the cytoplasmic domains (of 26 and of 47 or 94 residues) of muFcγRIII and muFcγRII (with b1 and b2 alternative splicing transcripts) share any homology (88). Analysis of overlapping λ genomic clones of muFcγRII revealed at

least 10 exons and covering ~15 kb. (160). A novel cytoplasmic exon was also found (160), which if spliced directly to the extracellular exon D2, could result in a secreted form of the receptor.

The muFcγRII gene is expressed in both myeloid and lymphoid cells and undergoes differential splicing dependent on the cell type (88). The transcripts are identical except for a 47 amino acid insertion in the cytoplasmic domain present in the muFcγRIIb1 transcript which is lacking in the muFcγRIIb2 transcript (88). MuFcγRIIb1 is expressed in both myeloid and lymphoid cells, while expression of muFcγRIIb2 is limited to macrophages and macrophage cell lines (88,155,161). A muFcγRII transcript of undetermined splice form is detected in a mastocytoma line (161). Ravetch et al. (88) have suggested that the splicing of muFcγRII transcripts may be developmentally regulated as the larger muFcγRIIb1 transcript was detected in more immature macrophage cell lines, while two of three mature macrophage lines did not express muFcγRIIb1; all of these lines did express muFcγRIIb2. As with muFcγRIII, the transcription of muFcγRII correlates with hypomethylation of a 5' region of the gene (161). Treatment of the thymoma BW5147 with 5-azacytidine results in hypomethylation and transcription of the muFcγRII gene while cotreatment with 5-azacytidine and the DNA methylating agent ethyl methanesulfonate abrogates the transcription (161).

MuFcγRIII

The structure and cloning of muFcγRIII was discussed above with that of muFcγRII. While the extracytoplasmic domains are quite nearly identical, important differences exist between their transmembrane and cytoplasmic domains which affect

their functional and signalling capacities. There is a highly conserved sequence in the transmembrane domains of muFcγRIII, huFcγRIIIA, and the α subunit of the high affinity rat FcγR$_\epsilon$RI. The transmembrane domains of these receptors each share a nearly identical stretch of ten amino acids, including a charged aspartyl residue in the middle of the lipid bilayer (24,88). Expression of the muFcγRIII gene, as for the huFcγRIIIA gene, is dependent on coexpression of the γ chain (109), and this receptor is the only FcγR expressed in NK cells where FcγRIIIA mediates antibody-dependent cell--mediated cytotoxicity (ADCC) (162). It is expected that the signalling and functions of muFcγRIII will be found to parallel those of huFcγRIIIA.

The muFcγRIII gene is expressed in macrophages and macrophage lines, a mastocytoma line, NK cells, and mesangial cells (155,162-165). Transcription of the muFcγRIII gene correlates with lack of methylation of specific sites in the 5' untranslated region of the gene, and the prevention of methylation of these sites (using the nucleotide analogue 5-azacytidine) results in expression of this gene in the thymoma cell line BW5147 (164). Two reports confirm that the muFcγRIII gene is in fact translated and expressed on the cell surface (163,164); both groups utilized an FcγRIII-specific anti-peptide antisera. The muFcγRIII protein has a M_r on SDS-PAGE of 45-55 kDa (152). The effect of a panel of lymphokines on muFcγRIII expression was studied (163) using the J774a cell line which does not express either muFcγRII message (155). Only IFNγ and interleukin 6 (IL6) resulted in increased binding of mAb 2.4G2 (163). IFNγ treatment resulted in increases in both muFcγRIII mRNA and protein, while IL6 treatment increased muFcγRIII mRNA but

not protein. In addition, IL6 treatment resulted in the expression of a mRNA species of higher mobility than the muFcγRIII message; this novel mRNA species was detectable on Northern blots with a probe specific for the extracellular domain of muFcγRII/III, but was not detectable with probes specific for the muFcγRIII or muFcγRII genes, leading to the proposal of the existence of a fourth form of muFcγR which possesses the 2.4G2 epitope (163). The presence of other muFcγR genes is suggested by the isolation of a cDNA clone which shares amino acid homology with muFcγRIII and muFcγRII (160).

Regulation of FcγR expression and function

Effects of cytokines and hormones

FcγRI (CD64) expression is regulated by interferon-γ, which increases the expression of FcγRI on monocytes and myeloid cell lines from 2- to 10-fold (166-168). In addition, IFN-γ (50 ng/ml) treatment of neutrophils, which normally do not express huFcγRI, resulted in the expression of $\sim$13,600 FcγRI molecules per cell (167). *In vivo* doses of IFN-γ (0.1 mg/m^2) resulted in elevated FcγRI expression on monocytes, measured by binding of fluorescein isothiocyanate (FITC)-IgG (169). Streptococcal pharyngitis can result in modest elevations of FcγRI number on monocytes and more dramatic levels on neutrophils (170). These results, however, were not correlated with elevated levels of IFN-γ, suggesting that other factors were responsible for the FcγRI elevation. HL-60 cells can be induced to express Fc$_\alpha$R by differentiating agents such as retinoic acid, DMSO, and calcitriol (171). However, IFN-γ decreased the expression of Fc$_\alpha$R in these cells.

Glucocorticoid therapy decreases FcγRI expression on monocytes *in vivo* (172) but is reported to have no effect *in vitro* (173). Decreased expression of FcγRI on monocytes was seen in patients treated for ITP with Danazol, a mildly androgenic steroid and correlated with the improved platelet counts seen in these patients (174). Dexamethasone augments the IFN-γ stimulation of FcγRI expression on monocytes (173,175), but abrogates the effect of IFN-γ on HL-60 cells and neutrophils (175,176).

Monocytes usually have little FcγRIIIA (177), although there is a small percentage of monocytes that express FcγRIIIA (178). However, incubation of fresh monocytes with 10 ng/ml of transforming growth factor β (TGF-β) for 24 hr results in FcγRIIIA expression on 40-80% of monocytes (179). There seems to be a non-responding population of monocytes in the population of cells purified by elutriation, suggesting possible monocyte heterogeneity. Monocyte FcγRI and FcγRII levels were unaffected by TGF-β. The rapid and striking induction of FcγRIIIA on monocytes by TGF-β has important implications for inflammation and wound healing. Other cytokines, including GM-CSF, IL-3, TNF, IL-4, and IL-6 had no effect on receptor number of either FcγRI, FcγRII, or FcγRIIIA (179).

There may be other factors that modulate transcription and/or translation of specific FcγR genes *in vivo*. For example, murine macrophages isolated from the peritoneal cavity of mice infected with BCG do not express FcγRIII. Treatment of the J774a macrophage cell line with IFN-γ resulted in increased expression of muFcγRIII (163). In addition, phorbol esters and dibutyryl CAMP have been shown to stimulate FcγRII expression and phagocytosis of erythrocytes sensitized with IgG

(EIgG) in U937 cells, although there was no stimulation by oleoyl-acetyl-glycerol (180). However, elevation of cAMP in neutrophils by treatment with forskolin markedly inhibited phagocytosis of IgG opsonized particles (181). It is difficult to compare these results obtained with different agonists in different cells.

IL-4 has clearly been shown to regulate FcγR function in murine B cells. IL-4 inhibits the ability of murine B cells to bind immune complexes, but does not significantly downregulate the absolute number of FcγRII molecules on the cell surface, as determined by anti-FcγR mAb binding (182). The inhibition by IL-4 might reflect altered mobility of the FcγRII on the plasma membrane, reducing the ability of immune complexes to cooperatively bind to FcγRII molecules. The ternary complex of rabbit anti-μ IgG binding to sIgM and FcγRII on B cells inhibits B cell proliferation (2). Treatment with IL-4 overcomes the rabbit anti-μ IgG inhibition, but the mechanism remains unclear (183).

Several cytokines can potentiate ingestion and the superoxide burst triggered by immune complexes without altering receptor number. GM-CSF, which does not change neutrophil FcγRII expression (179), enhances the production of superoxide by both neutrophils and monocytes in response to anti-FcγRII mAb crosslinking and stimulation by formyl-met-leu-phe (fMLP), without a change in the magnitude of the $[Ca^{2+}]_i$ flux (184,185). TNF-α (186,187) similarly will prime neutrophils for enhanced superoxide production following stimulation by fMLP. These results are in agreement with others (188) showing that GM-CSF enhances cytotoxic potential of ADCC by neutrophils and eosinophils without altering FcγRII expression.

Release of cytokines following ligation of FcγR

The binding of immune complexes to FcγRs also triggers the release of cytokines that may participate in inflammatory reactions. Crosslinking of NK cell FcγRIIIA triggers the activation of transcription and secretion of TNF-α, lymphotoxin, and IFN-γ (189,190). Similarly, macrophages can be induced to secrete TNF-α after crosslinking of FcγRI. FcγRII can also function as an inducer of TNF-α, but does so effectively only after protease digestion, which activates the receptor (191,192), as discussed below. This provides a degree of control for the elaboration of potentially deleterious cytokines, restricting optimum secretion to the locus of inflammation and hydrolase release.

Functions and Signalling by FcγR

Dissection of the functions and signalling pathways mediated by individual receptors was facilitated by the development of various monoclonal anti-FcγR antibodies. These reagents, however, still fail to distinguish amongst receptor isoforms of a given subclass, and even between murine subclasses II and III. The cloning of the FcγRs has enabled the unequivocal study of individual receptors. While redundancy of functions among FcγRs exists, the signalling mechanisms may be still be quite different amongst some FcγRs, insofar as they possess varied transmembrane and cytoplasmic domains. An intriguing homology exists between huFcγRIIA (and C), and the gamma subunits associated with huFcγRIIIA and the high affinity IgE receptor, Fc$_\epsilon$RI. The cytoplasmic domains of these molecules share a motif which is also present in subunits of the T and B cell antigen receptors. This motif was originally recognized by Reth (26) and the various receptors have

been termed *multichain immune recognition receptors* (MIRR) by Keegan and Paul (27). In this section, the known functions and signalling mechanisms of various FcγRs will be presented, followed by a discussion of the implications and recent developments concerning the MIRR motif. In this regard, the paradigms presented by the well studied T cell receptor (TCR) and Fc$_\epsilon$RI systems will be essential guideposts directing FcγR research.

Crosslinking of huFcγRI (by either ligand complexes or anti-huFcγRI mAbs) results in a number of functional responses; monomeric interactions have not been conclusively shown to generate any of these responses. In contrast to immune complexes which are endocytosed rapidly (193-195), monomeric ligand is not internalized or degraded through huFcγRI (195). These results imply that huFcγRI, when occupied by monomeric ligand, does not recycle (195). Similar results had been found with muFcγRII/III. Again, the fate of FcγR on the plasma membrane depends upon the valence of the ligand. The binding of IgG-sensitized erythrocyte ghosts to macrophages cleared $>50\%$ of muFcγRII/III from the plasma membrane (196). Once internalized with immune complexes and delivered to lysosomes the muFcγRII/III was rapidly degraded, with a T½ of <2 h compared to a T½ of 10 h in the absence of ligand (196,197). Likewise, FcγRII/III bound by multivalent preparations of mAb 2.4G2 Fab was transported to the lysosomes and degraded (198,199). However, FcγRII/III occupied with monovalent Fab fragments of mAb 2.4G2, was internalized, transported to endosomes, and rapidly delivered back to the surface (198). Upon binding of monomeric IgG, both FcγRII/III and ligand were recycled to the plasma membrane (193,195).

Crosslinking of huFcγRI using mAb 32 with a secondary anti-IgG reagent results in superoxide production (39). Continuous superoxide production via huFcγRI is dependent on continuous *de novo* formation of crosslinked huFcγRI (200). HuFcγRI on monocytes, macrophages, and IFN-γ treated neutrophils mediates phagocytosis of erythrocytes coated with heteroantibodies composed of Fab fragments of anti-huFcγRI mAb and Fab fragments of anti-erythrocyte antibody (201).

Recent work has shown that, with the exception of huFcγRIIIB, all three FcγRs are capable of ADCC towards target cells. The ADCC response is dependent on effector cell type and maturity, as well as the target cell type (202-204). Inflammatory mediators may also be important, since exogenous C1q reconstitutes FcγR-mediated ADCC and phagocytosis in mouse peritoneal macrophages (205). HuFcγRI on monocytes and macrophages effects ADCC of both hybridoma and erythrocyte targets. IFN-γ treatment augments huFcγRI-mediated ADCC of monocytes and induces that of neutrophils (168,202,203,206). Studies of ADCC with putative effector cell lines are largely unsatisfactory. Myeloid cell lines HL-60, U937, and THP-1 are unable to kill either erythroid or hybridoma targets, though IFN-γ treatment of these lines resulted in cytotoxicity against erythroid targets. When further differentiated (by 2-day culture), THP-1 cells exhibited slight huFcγRI mediated cytotoxicity (203).

Monocyte FcγRII also can mediate ADCC, both of hybridomas bearing anti-FcγRII mAb (207) and of IgG1-coated erythrocytes (208). Eosinophil and neutrophil ADCC -- which is mediated exclusively by FcγRII -- is induced by

GM-CSF (188), which also primes the superoxide burst as discussed above (179). FcγRIIIB, the GPI-anchored form of CD16 expressed on neutrophils is thought not to participate in ADCC reactions. Neutrophils cannot kill an anti-FcγRIII bearing hybridoma, although they can lyse chicken erythrocytes coated with anti-CD16/anti-chicken erythrocyte heteroantibodies (188). FcγRIIIB ligation has been demonstrated to trigger the release of hydrolases, but apparently cannot stimulate a respiratory burst (209), which may explain the above results. FcγRIIIA, expressed on NK cells, mediates ADCC (210). The interaction is strengthened by LFA-1 (CD11a), and inhibited by anti-LFA-1 mAbs. There is some conflict as to the ADCC potential of macrophage FcγRIIIA. In one report, an anti-FcγRIII mAb had no effect on the ADCC activity of cultured monocytes against Eigg, whereas FcγRI and FcγRII were shown clearly to mediate ADCC in this system (211). However, peritoneal macrophages and monocytes, which express little FcγRIIIA, were capable of killing an anti-FcγRIII hybridoma (204).

In addition to the established mechanisms for ADCC -- release of oxidative products and cytotoxic factors -- phagocytosis of tumor cells may play an important role in ADCC (212,213). This is not detected by isotope release assays, since the labeled phagocytosed targets are not lysed. Hybridomas bearing surface mAbs reactive with both FcγRII (CD32) and FcγRIII (CD16) were efficiently phagocytosed by monocytes cultured in M-CSF. Even though only about 20% of cultured macrophages expressed FcγRIII by flow cytometry, to obtain efficient inhibition of phagocytosis of IgG-sensitized tumor cells both FcγRII and FcγRIII had to be blocked (214).

HuFcγRIIA has been demonstrated to mediate phagocytosis when transfected into COS-1, 3T6 murine fibroblasts, and the P388D1 murine macrophage--like cell line (58,59,215), but was not able to do so in CHO fibroblasts that presumably lack the required signalling apparatus.(215). HuFcγRIIB (b1 form), which does not contain the MIRR motif, was unable mediate phagocytosis in COS-1 (59). A deletion mutant of huFcγRIIA (Δ264) lacking the 17 carboxyl--terminal residues (including the ultimate YXXL of the motif) was still able to mediate phagocytosis of receptor--bound complexes, although this ability did not extend to opsonized erythrocytes. This result may reflect a dependence of membrane remodeling (necessary for the ingestion of large particles) on the generation of a $[Ca^{2+}]_i$ flux; the Δ264 mutant did not signal for a $[Ca^{2+}]_i$ flux. Additional deletions (of 30 and 74 amino acids) resulted in totally nonfunctional receptors in this system. Similar results were obtained by transfecting chimeras containing the cytoplasmic domains of huFcγRIIA and FcγRIIC into a TCR-negative cytotoxic T cell line as well as primary human monocytes. In both cell types, the FcγRIIA and FcγRIIC chimeras mediated $[Ca^{2+}]_i$ flux and directed cytotoxicity against appropriate targets. The huFcγRIIB chimeras (both b1 and b2 forms) were not functional. Deletion mapping identified a 36 amino acid domain which spanned the MIRR motif which was required for function. Mutation of each of the two tyrosines within this domain abrogated both $[Ca^{2+}]_i$ flux and cytolytic capacity of the receptors in both TCR^- T cells and in primary monocytes (216). This result contrasts with the mutational analysis of huFcγRIIA to be presented in this thesis. Analysis of huFcγRIIA in BHK-21 (baby hamster kidney cell) transfectants demonstrated that progressive deletions from the

carboxyl--terminus resulted in a gradual, not abrupt, decline in phagocytosis of receptor--bound complexes (217), which differs from the previous two studies just discussed (215,216). The internalization of FcγRs transfected into nonphagocytic cells may not always reflect the true molecular physiology of these phagocytic receptors, but rather a more general nonspecific endocytosis of large surface protein aggregates.

Several lines of evidence point to the central role played by neutrophil FcγRII in triggering of the oxidative burst. Neutrophils that lack expression of FcγRIIIB, isolated from patients with paroxysmal nocturnal hematuria (PNH) -- a stem cell defect in which GPI-anchored proteins are absent -- undergo a normal superoxide burst when stimulated with immune complexes (22). The blockade of FcγRII on neutrophils by mAbs inhibits the respiratory burst, which cannot be triggered by crosslinking of neutrophil FcγRIIIB alone (218). Cleavage of FcγRIIIB by elastase, leaving FcγRII intact, does not alter the superoxide burst (135), and as discussed previously, proteolysis of FcγRII may enhance the immune complex binding capacity of this receptor (93).

It is likely that crosslinking of FcγRII on neutrophils and macrophages can result in activation of several signalling pathways. For example, the superoxide burst triggered by crosslinking by an anti-FcγRII mAb, KuFc79, is totally inhibited by pertussis toxin, suggesting that a G_i protein is central in this signalling pathway (219), but release of lysosomal hydrolases by the same stimulus is inhibited only 50% by pertussis toxin. In contrast, pertussis or cholera toxin had no effect on neutrophil degranulation and O_2^- production triggered by IgG coated surfaces.

However, degranulation and O_2^- production was inhibited by pretreatment of the neutrophils with phorbol myristate acetate (PMA) (220). PMA is known to interfere with G protein-protein kinase C interactions in activation of neutrophils by fMLP (221). Crosslinking of neutrophil huFcγRII by surface bound IgG was shown to augment neutrophil GTP binding and GTPase activity that was not affected by pertussis toxin (220). These contradictory results need to be sorted out by further research.

The signal pathways that are engaged following immune complex binding may vary depending on the state of the cell. For example, dibutyryl cAMP inhibits by about 70% superoxide release and ingestion by neutrophils presented with EIgG but has little effect on the same parameters by neutrophils primed with a cytokine from mononuclear cells presented with EIgG or with phorbol ester. In a reciprocal experiment, ingestion and superoxide release by unprimed neutrophils was unaffected by H7, a protein kinase inhibitor, but phagocytosis and superoxide release by cytokine and phorbol ester primed cells were inhibited by H7 (96).

MuFcγRII and or muFcγRIII mediate a variety of functional responses, including endocytosis of multivalent muFcγRII ligands, *in vivo* immune complex clearance, and ADCC. Unfortunately, these early studies do not identify which particular receptor is involved as muFcγRIII, muFcγRIIb1, and muFcγRIIb2 are all recognized by mAb 2.4G2 and are coexpressed on macrophages (88,155). NK cells express FcγRIII, but not FcγRII, and mediate ADCC (168). MuFcγRIII is expressed in a TCR-γ/δ^+ subset of murine dendritic epidermal cells which mediate ADCC and express the γ subunit mRNA of the T cell receptor (222).

The abilities of monomeric or aggregated IgG and mAb 2.4G2 to inhibit *in vivo* clearance of immune complexes were studied (223). MAb 2.4G2 was a very potent inhibitor of clearance of immune complexes and of IgG sensitized erythrocytes (maximal effect seen at 4 ug/gm), but also somewhat inhibited nonspecific clearance of heat damaged erythrocytes (223). While mAb 2.4G2 administration also resulted in a depletion of complement (223), inhibition of clearance did not depend on complement mediated lysis of macrophages as the inhibition was still seen in a C5-deficient strain of mice (224).

Immune complexes stimulate macrophages to release arachidonic acid which is subsequently metabolized through either the cyclooxygenase or lipoxygenase pathways (225-227) to produce prostaglandins and leukotrienes. Na^+ influx is an early and required event following $Fc\gamma R$ aggregation for arachidonate release (228). The release is blocked with either cycloheximide or actinomycin D, suggesting that a labile protein is involved in the signal transduction. Both the Na^+ and protein synthesis requirements could be bypassed by the Ca^{2+} ionophore A23187. External Ca^{2+} was required for both immune complex and A23187 induced release, whereas PMA-triggered release was only 50% inhibited in Ca^{2+} free media and could be completely inhibited by buffering intracellular Ca^{2+} as well (228). It should be noted that these studies do not address the involvement of any of the three classes--I, II, or III--of murine $Fc\gamma Rs$.

Initial studies demonstrated the role of macrophage $Fc\gamma Rs$ in ADCC (229) and the primary mechanism of this cytotoxicity was shown to be oxidative and involved the release of H_2O_2 (230). ADCC of BCG--elicited macrophages was

inhibited 70% by mAb 2.4G2, demonstrating the involvement of muFcγRII/III (231). It is unlikely that it is the muFcγRIII subclass which is responsible, as a recent study indicated that BCG--elicited macrophages do not transcribe the muFcγRIII gene (163).

Recent studies subsequent to the cloning of the muFcγRII/III genes have begun to address the roles of these particular receptors. The absolute or relative abundance of muFcγRIII mRNA is increased by IFNγ in macrophage cell lines and macrophages, and this expression correlates with an increased phagocytic capacity (155,163). BCG--elicited macrophages do not express detectable levels of muFcγRIII mRNA and are poorly phagocytic (232), whereas phagocytically active (232) resident and thioglycollate--elicited macrophages do express muFcγRIII (163).

It is clear that the cytoplasmic domain of FcγRII plays a role in endocytosis. The muFcγRIIb2 receptor transfected into CHO cells efficiently directs endocytosis via coated pits to the lysosomal compartment relative to the FcγRIIb1 splice variant, which has a 47 amino acid insertion in the cytoplasmic domain and is expressed primarily in B cells (233). However, both forms of the receptor will direct *Toxoplasma gondii* opsonized with IgG to lysosomes (234). A cytoplasmic domain deletion mutant is inactive in this respect. The muFcγRIIb1 splice variant is expressed predominantly on the apical plasma membrane of MDCK cells, whereas the muFcγRIIb2 variant is found on the basolateral aspect.

In contrast, recent studies suggest that the functions of muFcγRII may be quite circumscribed. MuFcγRIIb1 cannot mediate endocytosis in a B cell line, although it does cap when aggregated. MuFcγRIIb2 is not normally expressed in

lymphocytes, but when transfected into an FcγR-negative B cell line, it was capable of endocytosis. Both the b1 and b2 forms of muFcγRII were capable, when coaggregated with surface Ig, of inhibiting the B cell activation normally induced by aggregation of surface Ig alone. The domain required for this modulation, residues 18-31 of the cytoplasmic domain of muFcγRIIb2, was the same as that required for endocytosis. This domain is also present in b1, which is capable of modulation of B cell activation but not endocytosis. Thus two overlapping functional domains may be present in this sequence (235).

While both muFcγRII and III are expressed on murine mast cells, the degranulation response induced in these cells by crosslinking with the mAb 2.4G2 (236) may be mediated solely through muFcγRIII. Only muFcγRIII, but not muFcγRIIb1 or b2, was able to mediate serotonin and TNF-α release when transfected into a rat basophilic leukemia cell line (RBL-2H3) (236,237). Identical results were obtained when $[Ca^{2+}]_i$ flux, phosphoinositide hydrolysis, release of arachidonate metabolites, and protein tyrosine phosphorylation were assessed (125). The tyrosine phosphorylation response may be at least partially dependent on the presence of the β subunit in addition to the γ subunit required for FcγRIII expression. The presence of the β subunit was not required, however for FcγRIII-γ expression, nor was β needed for $[Ca^{2+}]_i$ flux and PI hydrolysis mediated by the FcγRIII-γ complex (124). It seems likely, but remains to be demonstrated if muFcγRIIb2 function in macrophages is limited to phagocytosis. The structural basis for the limitation of function of muFcγRII may be due to the incompleteness of the MIRR motif in these receptors: instead of YXXL, the carboxyl--terminal tyrosine

sequence in the muFcγRIIb1 and b2 cytoplasmic domains is YXXXI. The findings of Odin *et al.* (215) lend support to this hypothesis. A deletion mutant of huFcγRIIA, Δ264, which lacked the carboxyl--terminal 17 amino acids including the ultimate YXXL sequence, mediated phagocytosis of receptor complexes but was unable to elicit a $[Ca^{2+}]_i$ flux and the tyrosine phosphorylation response was markedly impaired (215). Crosslinking of muFcγRIIb1, either in B cells or expressed in CHO or MDCK cells results in phosphorylation on serine. However, muFcγRIIb2, which lacks the phosphorylation site, is not labeled under the same conditions. The kinase responsible is inhibited by staurosporine and by prolonged culture of the cells in PMA, suggesting that protein kinase C is responsible (238).

While huFcγRII may be primarily or solely responsible for the superoxide burst, both huFcγRII and huFcγRIIB are involved in other neutrophil responses to FcγR triggering. There are many more huFcγRIIIB sites, 135,000 sites per neutrophil (98), than huFcγRII sites, ~10,000 (77). ADCC of chicken erythrocytes (CE), coated with heteroantibodies composed of Fab fragments of anti-CE and anti-huFcγR mAbs, was mediated through both huFcγRII and huFcγRIII on neutrophils (188). However, neutrophils could not kill an anti-huFcγRIII hybridoma cell line. Recent work, as discussed above, demonstrated that huFcγRIIIB can trigger the release of hydrolases but not a respiratory burst upon being crosslinked (209). This may account for the observed lysis of CE but not of hybridoma cells mediated through huFcγRIIIB.

The high density of huFcγRIIIB on neutrophils may serve to focus immune complexes on the cell surface where they can interact with and trigger huFcγRII. In

fact studies (72,239) suggest that huFcγRIII is involved in the initial adherence of neutrophils to IgG-coated erythrocytes. Likewise, huFcγRIIIB was essential for the binding of small immune complexes to neutrophils, whereas huFcγRII only weakly enhanced this binding (77). Yet, this essential binding role of huFcγRIIIB did not extend to large immune complexes, and neutrophils from patients with paroxysmal nocturnal hematuria , which only express 10% of normal levels of huFcγRIIIB, had normal metabolic responses to IgG-latex (77). A patient with SLE was found who did not express huFcγRIIIB on her neutrophils, due to a probable deletion of the huFcγRIIB gene (240). The patient's neutrophils did have reduced ability to rosette IgG-coated E, as suggested by earlier studies of neutrophil function (72,239). However, this patient did not exhibit any unusual susceptibility to bacterial infections, and the levels of other GPI-linked proteins and huFcγRII were normal. Eight other patients diagnosed with SLE had normal levels of huFcγRIIIB.

There has been some conflict about the role of the GPI-anchored FcγRIIIB molecule expressed on neutrophils. It is difficult to visualize how such a molecule would transduce signals, since there is no cytoplasmic or transmembrane domain, and the proposal has been made that FcγRIIIB serves to focus or concentrate immune complexes. However, crosslinking of FcγRIIIB triggers a $[Ca^{2+}]_i$ flux (241,242) and the release of hydrolases (209). The possibility that FcγRIIIB signals to neutrophils through FcγRII has been suggested, based on the observation that degranulation of neutrophils triggered by IgM anti-FcγR mAb that is specific for FcγRIII but not FcγRII can be inhibited by either anti-FcγRII or anti-FcγRIII mAb Fab fragments (243). The signalling may resemble GPI-anchored protein signalling of T cell

mitogenesis (see (244) for review). T cell mitogenesis stimulated via crosslinking of GPI-anchored molecules requires the GPI anchor (245) and a functioning Ti/CD3 complex (246,247). In addition, some authors contend that the GPI-anchored proteins must be allowed to aggregate by addition of antibody in solution, but this finding is controversial. Other evidence for interaction between neutrophil FcγRIIIB and FcγRII is the enhanced phagocytosis of anti-FcγRII Fab-coated erythrocytes following crosslinking of FcγRIIIB (248).

Another model for heterotypic signal transduction comes from study of CD2 signalling. Like the GPI-anchored molecules, CD2 signalling requires intact Ti/CD3 complexes (249). When CD2 was transfected into a mast cell line expressing FcγRII and FcγRIII, production of IL-6 could be demonstrated following addition of antibodies directed against different epitopes of CD2 ($T11_2$ and $T11_3$). The signalling was dependent on the interaction of the anti-CD2 mAbs with FcγR, because the stimulus was abolished by anti-FcγRII and anti-FcγRIII mAb 2.4G2 (181). Crosslinking of Fc$_\epsilon$RI also resulted in IL-6 synthesis. However, there were significant differences in cell signalling mechanisms, since engagement of the FcγR did not always trigger a $[Ca^{2+}]_i$ flux and Fc$_\epsilon$RI ligation did. It is not clear from these results whether FcγRII or FcγRIII is interacting with the crosslinked CD2, but FcγRIII is the favored candidate.

HuFcγRIIIA on NK cells mediates ADCC after crosslinking (210). Immune complex crosslinking of the receptor also induced transcription of the interleukin-2 receptor, IFN-γ, and TNF-α, all of which activate NK cell activity (189). Therefore, in addition to acting as a trigger for ADCC on NK cells, the activation of

huFcγRIIIA on NK cells also potentiates the Ig-independent, natural killer activity of NK cells. The ability of anti-LFA-1 (CD11a) antibodies to inhibit huFcγRIIIA mediated ADCC by NK cells suggests involvement of this adhesion receptor in effector cell-target cell apposition during NK-mediated ADCC (210). Similarly, in monocytes but not lymphocytes, blocking of LFA-1 inhibited ADCC mediated through crosslinking of huFcγRs (204).

Small subsets of NK cells express little or no huFcγRIIIA (250). The level of huFcγRIII expression may signify different developmental stages in the NK cell lineage. The low or non-huFcγRIIIA expressing NK cells proliferate more extensively in response to rIL-2 (251). The most mature stage, based on low proliferative ability, high abundance in blood, and high cytotoxic potential are NK cells expressing abundant huFcγRIIIA.

A number of studies have demonstrated that huFcγRIIIA on macrophages in the spleen and on Kupffer cells is the primary receptor responsible for clearance of large immune complexes. In chimpanzees, the anti-huFcγRIII mAb 3G8 inhibited clearance *in vivo* of autologous erythrocytes coated with antibody directed against a minor blood group antigen (7). MAb 3G8 has been tested as a potential therapeutic treatment for individuals with immune thrombocytic purpura, a disease in which patients secrete high levels of anti-platelet antibody (252). Treatment of one patient resulted in a dramatic rise in platelet levels, returning to normal levels within two weeks. Unfortunately, a second treatment gave a much less dramatic response. Sensitization to the murine mAb may reduce its effectiveness.

HuFcγRIIIA on cultured monocytes is biochemically indistinguishable from that of NK cells (211). There are conflicting reports about whether huFcγRIIIA is a trigger molecule for ADCC by macrophages. Addition of anti-huFcγRIII mAb, CLB-FcR-gran 1, did not reduce the lytic activity of cultured monocytes against erythrocytes sensitized with 4 x 10^4 molecules per E of different isotypic switch variants (IgG1, IgG2a, and IgG2b) of a murine mAb or equal amounts of an IgG3 from a different murine hybridoma cell line (211). Steps were taken to be sure that experiments were done below the maximum lytic activity of the other huFcγR on cultured monocytes in order to be able to detect inhibition by mAb CLB-FcR-gran 1. However, peritoneal macrophages, even fresh monocytes, were clearly able to kill an anti-FcγRIII bearing hybridoma cell line (HC 3G8) (204). This was the first evidence that huFcγRIIIA may be functionally detectable on fresh monocytes. The discrepancy in killing ability may be due to the different target cells and mAbs used in each study.

Calcium flux in response to FcγR crosslinking

Crosslinking of FcγR in most cases seems to trigger an increase in $[Ca^{2+}]_i$. Crosslinking of both FcγRI and FcγRII on U937 cells resulted in an increase in $[Ca^{2+}]_i$. The crosslinking of FcγRII expressed on platelets (253,254), eosinophils (255), and neutrophils (185) leads to elevations in $[Ca^{2+}]_i$, and in the production of inositol phosphates, reflecting the activation of a phosphatidylinositol-specific phospholipase C. Another study reported that huFcγRIIA was not able to trigger a $[Ca^{2+}]_i$ flux in neutrophils (242). This conflicting result may be due to differences in the activation state of the PMNs used or may reflect differences in antibody reagents

that may have interacted with other FcγRs through their Fc portions. Activation of NK cell FcγRIIIA (190,256) and mesangial cell FcγRIII (257) lead to a prompt increase in $[Ca^{2+}]_i$, and release of inositol phosphates. There is some conflict as to whether the GPI-anchored neutrophil FcγRIIIB triggers a $[Ca^{2+}]_i$ flux. Three groups (241,242,248,258) report increases in $[Ca^{2+}]_i$ upon crosslinking of neutrophil FcγRIIIB but others (cited as unpublished results in (12)) find no $[Ca^{2+}]_i$ signal. In contrast to these results, the ternary complex of anti-IgM IgG binding to sIg and the FcγRII of murine B cells blunts the $[Ca^{2+}]_i$ influx associated with crosslinking of sIg (259).

FcγRs and phosphorylation events

FcγRIIIA expressed on macrophages is associated with a disulfide linked γ chain dimer (109) that is shared by the high affinity $Fc_\epsilon RI$ present on mast cells and basophils. FcγRIIIA on NK cells is associated with both the γ chain and a related molecule, ζ (113,114), which is also found in the Ti/CD3 complex (121). These two chains can form either homo- or heterodimers, and in the absence of these associated peptides FcγRIIIA expression is reduced by 50-100-fold (109). Crosslinking of FcγRIIIA on NK cells results in phosphorylation on tyrosine of the ζ chain (111,121). This phosphorylation is specific for FcγRIIIA activation. ADCC but not natural killing of target cells by NK cells induces ζ chain phosphorylation on tyrosine (111), although other NK cell proteins are phosphorylated on tyrosine during natural killing target cell recognition (260). In fact, the tyrosine kinase inhibitor Herbimycin A (261-263) has been shown to inhibit both ADCC as well as natural killing of nonopsonized targets by NK cells (125). The kinetics of

$Fc\gamma RIIIA\zeta$ phosphorylation are rapid, occurring within 5 min, and transient, returning to baseline by 1 hr. The similarities in response and kinetics to the ζ phosphorylation of the Ti/CD3 complex (117), suggest that $Fc\gamma RIII$ and the Ti/CD3 complex may have similarities in signal transduction. TCR signalling is also inhibitable with Herbimycin A (264). However, as discussed above, crosslinking of the NK $Fc\gamma RIIIA$ receptor also triggers the $[Ca^{2+}]_i$ flux, and the release of inositol phosphates. The order of the signal sequence is far from clear, and the identity of the kinases that mediate these phosphorylation events also needs clarification.

Some of the MIRR receptor subunits containing the D/E-X_7-D/E-X_2-Y-X_2-L-X_7-X_2-Y-X_2-L motif are capable of signal transduction. The ζ chain of the CD3 complex of the TCR has been most extensively studied. Initial studies of a ζ-negative T cell hybridoma (119), as well as reconstitution experiments with wild type or deletion mutants of zeta (265) demonstrated that ζ is required for antigen signalling of the TCR. A CD8 chimera containing the cytoplasmic domain of ζ was itself able to elicit generation of inositol triphosphate, tyrosine phosphorylation, interleukin-2 release, and CD69 expression (266). Similarly, a CD4-ζ chimera demonstrated cytolytic capability against an HIV-1 envelope glycoprotein--expressing target cell (267). Deletion studies first indicated that only one of the three copies of the motif present in ζ is necessary to transduce a signal, albeit a reduced response (268). Further work has demonstrated that an 18 amino acid stretch of ζ which contains the acidic tyrosine-leucine motif is sufficient to signal for a calcium flux and cytolysis; the two tyrosines were shown by site--directed mutagenesis to be crucial (30). However, two earlier studies demonstrated that mutation (to phenylalanine) of

any of the six tyrosine residues of the cytoplasmic domain of the ζ chain did not alter the ability of these mutants to signal for the production of interleukin-2 (IL-2) (29,265). These seemingly inconsistent findings may reflect the possibility that only a subset of the TCR-mediated responses are dependent on the tyrosine residues of the MIRR motif--containing chains. The ϵ subunit of the TCR/CD3 complex can also independently signal when expressed in a thymoma cell line in a chimeric interleukin-2 receptor α construct (269) and may explain the ability of TCR/CD3 complexes to signal in cells not expressing or expressing a severe truncation of ζ (268). As with ζ, the two tyrosine residues of ϵ (as well as some adjacent residues) were found in one study to be critical for signal transduction (269).

Heterodimers of the zeta family are also expressed in the TCR. The ζ-η heterodimer is expressed in 10-20% of TCR (270) and may be responsible for the coupling of the TCR to phosphoinositide hydrolysis (271). The ζ-γ heterodimer has also been demonstrated in T cells, but the γ-γ homodimer has only been shown in Fc receptors (both FcγRIII and Fc$_\epsilon$RI). In Fc$_\epsilon$RI, the γ-γ homodimer may be substituted with the ζ-ζ homodimer (272). The signalling and functional differences that may result from these varied zeta family dimers is an area of active study.

Signalling events following the ligation of Fc$_\epsilon$RI are well studied. Early events include the activation of phospholipase C (phosphoinositol--specific, PI-PLC) resulting in the generation of inositol phosphates (IP_3) and diacylglycerol (DAG), leading to the release of intercellular stores of Ca^{2+} and the activation of protein kinase C (PKC). Additionally, activation of phospholipases D and A2 and Ca^{2+} influx (from extracellular media) occur [reviewed in (273,274)]. The $\alpha\beta\gamma_2$ complex

of $Fc_\epsilon RI$ is predicted to have seven transmembrane spanning domains (with four provided by the class III β subunit) (104). While this structure suggests that the receptor interacts with a G protein, to date only indirect evidence, which is not entirely consistent, has been obtained [as detailed in refs. (275,276)].

$Fc_\epsilon RI$ crosslinking results in phosphorylation of both receptor subunits as well as multiple cellular proteins. The β subunit is phosphorylated on tyrosine and serine, while the γ subunit is phosphorylated on tyrosine and threonine (277-279). The receptor phosphorylation is rapidly reversible upon receptor disengagement (13), as is the association of the phosphorylated receptor with pp125 which is phosphorylated on serine in response to $Fc_\epsilon RI$ crosslinking (278). One of the most prominent proteins phosphorylated on tyrosine upon $Fc_\epsilon RI$ ligation is pp72 (276,280-282). The tyrosine phosphorylation of pp72 is an early event (maximal by 1 min) occurring independently of $[Ca^{2+}]_i$ flux and PKC activity (280) and is not affected by stimulation or inhibition of G proteins (276). The phosphorylation of pp72 was blocked by either RG 50864 (281) and genistein (282), and this inhibition correlated with decreased serotonin and histamine release, whose release from mast cells or basophils via degranulation is one of the end results of $Fc_\epsilon RI$ activation. The phosphorylation of another protein, pp110, which exhibits slower kinetics than pp72, requires Ca^{2+} influx and PKC activity (282) and this phosphorylation may be required but is not sufficient for degranulation (281,282).

An exciting and revealing development in the understanding of $Fc_\epsilon RI$ signal transduction has been the demonstration by several groups that $Fc_\epsilon RI$ crosslinking results in tyrosine phosphorylation of PI-PLC-γ1 (275,279). Activation of PLC-γ1

is known to occur through phosphorylation of multiple tyrosine residues without the participation of any G protein (283-286). A recent report in apparent conflict with these results is the inability of even high concentrations of genistein to affect neither $Fc_{\epsilon}RI$-mediated phosphatidylinositol hydrolysis nor the generation of the second messenger inositol (1,4,5)-triphosphate (276). While additional studies are clearly necessary to clarify these discordant results, it is possible that a genistein--insensitive tyrosine kinase is involved in the activation of PI-PLC-γ1. Indeed, another report showed that the tyrosine phosphorylation of PI-PLC-γ1 involves a different pathway than that of the receptor and other cellular proteins such as pp72 and pp110. In this study, the tyrosine phosphorylation of PI-PLC-γ1 as well as phosphatidylinositol hydrolysis, $[Ca^{2+}]_i$ flux, and serotonin release were found to be inhibited by micromolar concentrations of phenylarsine oxide (PAO), while other tyrosine phosphorylations, including the receptor itself were not inhibited (287). In micromolar concentrations, PAO is known to inhibit tyrosine--specific phosphatases including CD45 (288). This is intriguing as CD45 is probably an essential element in the signal transduction pathways of other MIRR, as will be further discussed below (126,288-290). Recently, the $Fc_{\epsilon}RI$ has been shown to undergo multiubiquitination upon receptor crosslinking. This is rapid and reversible, but occurs only on aggregated receptors. Moreover, it is independent of the phosphorylation status of individual receptor subunit molecules, *i.e.* both phosphorylated and nonphosphorylated ubiquitinated forms of receptor subunits (β and γ) are observed (291).

Tyrosine phosphorylation of PI-PLC-γ1 has also been demonstrated in the TCR (292) and BCR (293) systems. Phosphopeptide map analysis showed that the major sites of tyrosine and serine phosphorylation of PI-PLC-γ1 in a human T cell line (Jurkat) stimulated with anti-CD3 antibodies were the same as in EGF or PDGF-treated cells (294). In parallel to the PAO results discussed with the $Fc_{\epsilon}RI$, the oxidant *N*-ethylmaleimide, which inhibits TCR-mediated $[Ca^{2+}]_i$ flux and IL-2 production, inhibited the tyrosine phosphorylation of PI-PLC-γ1 (295). Activation of PI-PLC-γ1 via stimulation of the human muscarinic receptor, subtype 1, which is G protein linked, did not induce the tyrosine phosphorylation of the enzyme (292). Serine phosphorylation of PI-PLC-γ1 by PKA (cAMP-dependent kinase) and PKC may serve to modulate the interaction of the enzyme with tyrosine kinase or phosphatase enzymes (296). Ligation of membrane IgM (BCR) on two human lymphoblastoid cell lines (Daudi and the L4 subclone of Ramos) also induced tyrosine phosphorylation of PI-PLC-γ1 (293). However, another study found that while a minimal increase in tyrosine phosphorylated PI-PLC-γ1 occurred in the murine B cell line Bal17 upon anti-IgM treatment, increases of tyrosine phosphorylated PI-PLC-γ2 were detectable in both B cell lines Bal17 and WEHI-231, as well as in normal resting murine B cells (297). These results illustrate that care must always be taken when interpreting results obtained in transformed cell lines. HuFcγRI, which does not contain the MIRR cytoplasmic motif, and huFcγRII, which contains a variant of the motif, are each able to mediate the tyrosine phosphorylation of PI-PLC-γ1 in a human monocyte line, U937.

Pretreatment with Herbimycin A abolished this phosphorylation as well as phosphatidylinositol (PI) turnover (298).

In addition to PI-PLC, phospholipase D (PLD) may play a pivotal role in the release of mediators of inflammation (histamine, leukotrienes, and arachidonic acid) from mast cells. PLD acts at the terminal phosphodiester groups of phospholipids, primarily phosphatidylcholine (PC) to yield phosphatidic acid (PA). The PA can be subsequently dephosphorylated by PA phosphohydrolase (PAPase) to yield diacylglycerol (DAG). DAG is important both as an activator of PKC and as a substrate source for the production of arachidonic acid. PC is a quantitatively more important source of DAG in mast cells triggered through $Fc_{\epsilon}RI$ (299). Pharmacological inhibition of the production of PLD-derived DAG either with ethanol (which results in phosphatidylethanol instead of PA) or with *d,l*-propranolol (which blocks the PAPase conversion of PA to DAG) resulted in the inhibition of $Fc_{\epsilon}RI$-mediated release of histamine and arachidonate metabolites (300). PA, produced by PLD action, is also important in the stimulation of the enzyme complex of neutrophils responsible for the production of superoxide and may function in response to chemotactic peptide (301). The role of PLD in $Fc_{\gamma}R$ signalling remains to be determined.

Following MIRR-stimulated phosphorylation, dephosphorylation occurs rapidly and this suggested obvious involvement of phosphatases in these signalling events. The role of phosphatases appears to be more complex however, than simply turning off a phosphorylation signal. A tyrosine phosphatase, CD45, is required for TCR signalling, and CD45 may modulate the cellular response to $Fc_{\epsilon}RI$ and BCR.

The CD45 family, whose expression is limited to hematopoietic cells, is composed of eight mRNA isoforms (secondary to alternate splicing) encoding integral membrane proteins of M_r 180,000 - 220,000. These isoforms vary in their extracellular domains but possess essentially the same phosphotyrosine phosphatase (PTP) activity in their intracellular domain (302,303). T cells which lacked expression of CD45 were unable to proliferate in response to CD3 crosslinking, but a CD45-expressing revertant was able to respond (304). Aggregation of CD45 on normal human T cells also inhibited their proliferative response to anti-CD3 as well as to IL-2 (305). Transfection of CD45 into a $CD45^-$ human leukemic cell line restored a proliferative response to TCR stimulation. In addition, stimulation of inositolphosphate metabolism was possible in the $CD45^-$ cells via transfection and stimulation of the human muscarinic type 1 receptor which activates a PLC (but *not* PLC-γ1) through a G protein. Thus, it was suggested that CD45 normally acts prior to the production of inositol phosphates, although the interaction of CD45 with PI-PLC-γ1 itself cannot be ruled out (289). CD45 has been shown to be associated with the TCR (306-307) and modulates the tyrosine kinase activity of $pp56^{lck}$ and $pp59^{fyn}$ (308-311). In a $CD45^-$ plasmacytoma cell line, the ability to mobilize Ca^{2+} when surface mIgM of the BCR was ligated was restored by transfection of CD45. Moreover, CD45 was shown to modulate the phosphorylation of the Ig-α and Ig-β subunits both *in vitro* and *in vivo* (126). Treatment of human basophils with anti-CD45 mAb inhibited the Fc_ϵRI-mediated release of histamine (290). The exact nature of the role of CD45, either as a regulatory or direct component of the various MIRR signalling pathways, remains a topic of current study. The common

involvement of CD45 with these various receptors underscores that MIRR represent close variations on a theme of signal transduction.

A clearly emerging pattern of MIRR signalling is that activation of at least one nonreceptor tyrosine kinase is the earliest cellular response to receptor crosslinking. The kinetics of tyrosine phosphorylation preceded phosphatidylinositol (PI) hydrolysis induced through MIRRs (312) and inhibitors of tyrosine kinase function (genistein or Herbimycin A) completely block the ability of the TCR to stimulate PI turnover (264,313). The kinetics of tyrosine phosphorylation stimulated via the BCR, $Fc_{\epsilon}RI$, and $Fc\gamma Rs$ is also rapid and transient and appears to be a more proximal event, or at least is independent of $[Ca^{2+}]_i$ flux or PKC activation (314,315). As was discussed above, tyrosine kinase inhibitors results in the inhibition of $Fc_{\epsilon}RI$-mediated degranulation (281,282) and $Fc\gamma RIIIA$-mediated $[Ca^{2+}]_i$ flux, PI turnover (315), ADCC and NK killing (125). Attempts to identify a kinase involved in MIRR signal transduction has been a rapidly enlarging field of study.

Again, the TCR system is the most extensively studied to date. Early progress ensued when a member of the Src family of tyrosine kinases, $p56^{lck}$ was found to be associated noncovalently with CD4 and CD8 (316-319). CD4 and CD8 are expressed on T cells and recognize class II and class I MHC molecules, respectively. Antigen is *presented* to T cells in physical association with either MHC class I or II, and this antigen-MHC complex is bound by both the TCR and the appropriate CD8 or CD4 molecule, bringing the TCR in close approximation with the CD4 or CD8 protein (320). Crosslinking of CD4 induced activation of $pp56^{lck}$ and their association was required for activation in a T cell clone (321,322).

However, TCR ligation did not activate lck (321) and TCR- and CD4- induced tyrosine phosphorylation patterns of cellular proteins differed (323). Thus while lck may not be the very proximal transducer of the TCR signal, it nevertheless is positioned to play an important regulatory role. That this regulatory role may be in fact required for TCR signalling was demonstrated in a recent study of a T cell line which was defective in functional lkc expression (secondary to aberrant mRNA splicing). The lck$^-$ mutant was unresponsive to TCR crosslinking, but transfection of a *lck* cDNA enabled complete TCR responses, including tyrosine phosphorylation and $[Ca^{2+}]_i$ flux. In addition, this transfection restored the *in vitro* kinase activity which normally coprecipitates with and phosphorylates the ζ subunit (324). Another candidate for mediation of the TCR signal is p59^{fyn}, which coprecipitates with the TCR and is also a member of the Src family (325). Very recent work has identified a protein tyrosine kinase (PTK) of 70,000 M_r, termed ZAP-70, which is phosphorylated and is associated with the ζ subunit following TCR stimulation (28,326,327). A homologous PTK, syk, of 72,000 M_r has been cloned from porcine splenic cells (328).

Src family kinases are likely to mediate signalling for a variety of MIRRs. Preliminary studies suggest that $Fc_\epsilon RI$ activates the lyn and yes kinases (325,329), and stimulation of $Fc\gamma RIIIA$ results in lck activation (315). In B cells, stimulation of the antigen receptor (via either sIgM or sIgD) also results in tyrosine phosphorylation of cellular target proteins, including at least the Ig-α receptor component as well (314,330,331). Again Src family kinases are emerging as potential signal transducing molecules. Blk is solely expressed in B-lineage cells

(332), and lyn, which is preferentially expressed in B cells, could be coprecipitated with sIgM of the BCR (333). A more recent study has shown that three Src family kinases, blk, fyn, and lyn, are activated by the BCR and coprecipitate with sIg. In this study, the activation of blk was more marked than of the other two kinases (334).

FcγRIIA, when expressed in a murine macrophage--like cell line, P388D1, mediates phagocytosis, internalization of soluble immune complexes, and a Ca^{2+} flux (215). FcγRIIA expressed in COS-1 also mediates phagocytosis and crosslinking of this receptor induced tyrosine phosphorylation on a number of proteins, including FcγRIIA (58,128). Previous analysis of cytoplasmic truncation mutants revealed that the 17-amino acid carboxyl--terminal is required for phagocytosis and Ca^{2+} flux. A second region between residues 234 and 264 was necessary for the rapid internalization of cell surface--bound immune complexes (215). We have now examined the role in signal transduction of individual amino acids of the cytoplasmic domain between residues 234 and 263, including residues of the MIRR motif (335), and these results will be discussed in this thesis.

II STUDY OF A CHIMERIC HSA-muFcγRIIb2 RECEPTOR

Introduction

FcγRs differ from many other receptor classes, such as hormone and neurotransmitter receptors, in that crosslinking or immobilization of the receptors in the membrane by polyvalent immune complexes, rather than binding of ligand *per se* is the triggering event. Analogously, growth factor receptors undergo dimerization upon ligand binding. Monomeric interaction of ligand with any of the receptors of the MIRR family is *not* sufficient for signalling. This is true for both receptors with low affinity (such as FcγRII or CD32) and for receptors with high affinity. The paradigm for the latter class is Fc$_\epsilon$RI [see (10,12) for review]. Degranulation of mast cells or basophils does not occur until the crosslinking of Fc$_\epsilon$RI is initiated by the binding of multimeric IgE-antigen complexes. Reagents (such as anti-FcR antibodies) that crosslink the receptor by binding to epitopes other than those involved in the immunoglobulin Fc binding site are effective triggers [reviewed in (10-12,14,15,31,32,203,278,336-339).] The sufficiency of this nonphysiological crosslinking to induce signalling suggests that binding of ligand by itself does not result in transmission of a conformational change to the cytoplasmic or transmembrane domains. Anti-CD3 and anti-TCR antibodies are also able to induce signalling in various T cell systems (19,320). Anti-IgD, anti-IgM, or anti-IgG antibodies which crosslink the B cell receptor (BCR) elicit the same proliferative response normally induced by polyvalent antigen complexes (126,127,340). There may, however, exist preferred orientations of receptors engaged in signalling as is suggested by reports that mAbs directed against the β subunit of the Fc$_\epsilon$RI are not

capable of initiating signalling when used to crosslink the receptor (12). The exact degree of crosslinking which is minimally required to elicit a signal is not well studied for any of the MIRRs.

I wished to examine the role of receptor crosslinking for signalling and to specifically address the role of the extracytoplasmic domain. I hypothesized that the essential initial signalling event is receptor crosslinking, which is independent of any conformational change transduced through the external domain upon ligand binding. To test this hypothesis, I developed a chimeric model system in which the external ligand--binding domain of the murine FcγRII (b2 splice form) was replaced with human serum albumin (HSA). Anti-HSA crosslinking reagents were then utilized to assess the functional capacity of this receptor.

Results

A recombinant cDNA encoding the chimeric HSA-muFcγRIIb2 was inserted into the LK444 expression vector and transfected via a modification of the calcium phosphate--DNA coprecipitate technique into the functional murine macrophage--like cell line P388D1. Transfectants obtained using only the parent vector, LK444, served as a negative control cell line (P-0).

Transfectants were selected and maintained in media containing Geneticin (G418) and were screened for expression of the chimeric HSA-muFcγRIIb2 receptor by FACS analysis. Expression of the chimeric HSA-muFcγRIIb2 by the HSA-FcRII cell line is shown in Figure 4, and the mean peak channels are presented in Table 2. Cells were stained with either a goat $F(ab')_2$ anti-HSA or anti-IgD (negative control) antibody which was in turn detected by a FITC-conjugated $F(ab')_2$ rabbit anti-goat

IgG. Results with the negative control cell line, P-0, show that there is no difference in the background level of staining obtained with either the anti-HSA or anti-IgD reagent. There was also no significant difference in the mean peak channels obtained with unstained cells and cells stained with the FITC-conjugated secondary reagent alone when compared to the staining with anti-IgD reagent (data not shown) for either the positive HSA-FcRII cell line or the negative control cell line, P-0. These results indicated that the increased staining of the HSA-FcRII cell line with the anti-HSA reagent was due to surface expression of the chimeric HSA-muFcγRIIb2 receptor. While the level of expression was low ($\approx$20-25 mean peak channel difference above background fluorescence in most experiments), the precision of FACS analysis did enable study of the course of surface expression following receptor crosslinking.

Competitive inhibition of binding studies were undertaken to demonstrate the specificity of the anti-HSA staining results. Bovine, human, and murine serum albumin (BSA, HSA, MSA) (at 10 mg/ml) were used as competitive inhibitors of the anti-HSA antibody binding. Two concerns necessitated this study. The first concern was that bovine albumin in the tissue culture media may have adsorbed to the cell surface, resulting in the positive staining. This possibility was considered unlikely, however, as the control cells which were grown in the identical medium did not exhibit significant positive staining (see Fig. 4, top panel, and Table 2). The second concern was that expression of murine albumin or a murine albumin--like molecule may have been induced by the transfection procedure or may have been expressed normally by these immortal cells. There are several reports in the

Table 2 A. Expression of a chimeric HSA-FcγRII. The HSA-FcγRII and P-0 cell lines were stained as detailed in Materials and Methods and analyzed using a Coulter Epics Cell Sorter (FACS analysis). **B.** Competitive binding study. For the competitive iinhibition studies, bovine, murine or human serum albumin were present at a final concentration of 10 mg/ml in the α-HSA staining medium. Values obtained from α-HSA staining of the HSA-FcγRII and P-0 cell lines were taken as 100% and 0% receptor staining. Shifts in log fluorescence were converted to linear fold increases in fluorescence. The % of maximal receptor staining was calculated as:

(fold increase in the presence of inhibitor - 1)
÷ (fold increase in the absence of inhibitor - 1)

% Inhibition = 100% - the % of maximal staining in the presence of inhibitor

A. Expression of a Chimeric HSA-FcγRII

Cell Line	Antibody	Peak Channel
HSA-FcγRII	αHSA	151
"	αIgD	124
P-0	αHSA	131
"	αIgD	125

B. Competitive Binding Study

Experiment 1

Cell Line	Inhibitor	Antibody	Peak Channel	% Pos	% Inhib
HSA-FcγRII	0	αHSA	151	100	NA
"	BSA	"	148	81	19%
"	HSA	"	134	14.5	85.5%
"	0	αIgD	124		
P-0	0	αHSA	131	(0%)	
"	BSA	"	132		
"	HSA	"	139		
"	0	αIgD	125		

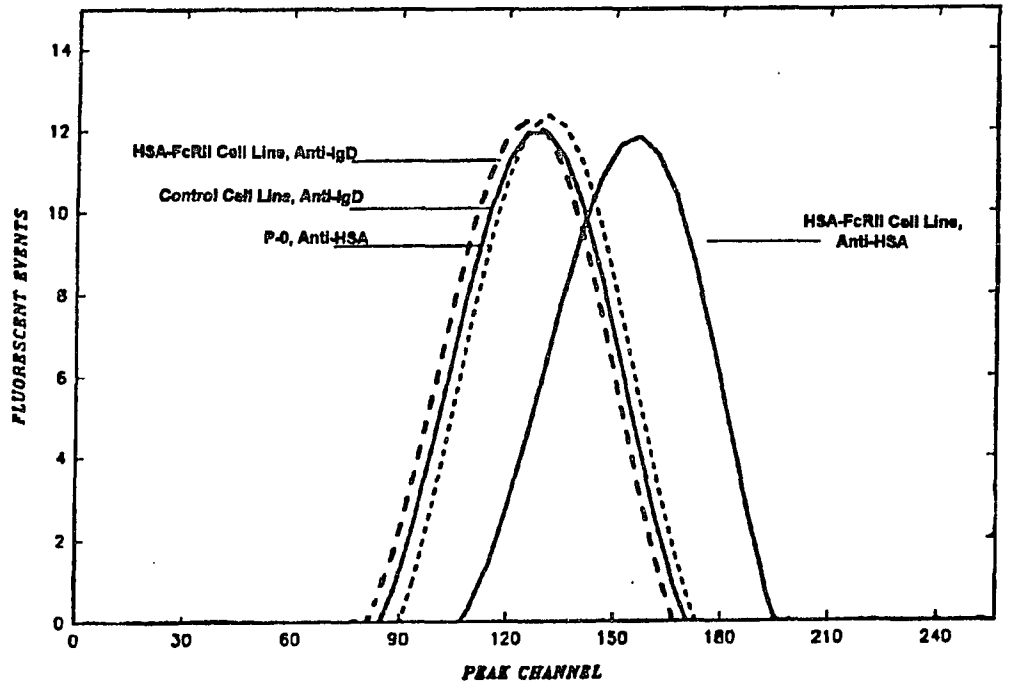
Experiment 2

Cell Line	Inhibitor	Antibody	Peak Channel	% Pos	% Inhib
HSA-FcγRII	0	αHSA	126	100	
"	BSA	"	125	94	6
"	MSA	"	120	66	34
"	HSA	"	107	0.1	99.9
"	0	αIgD	99		
P-0	0	αHSA	105	(0%)	
"	BSA	"	109		
"	MSA	"	107		
"	HSA	"	109		
"	0	αIgD	100		

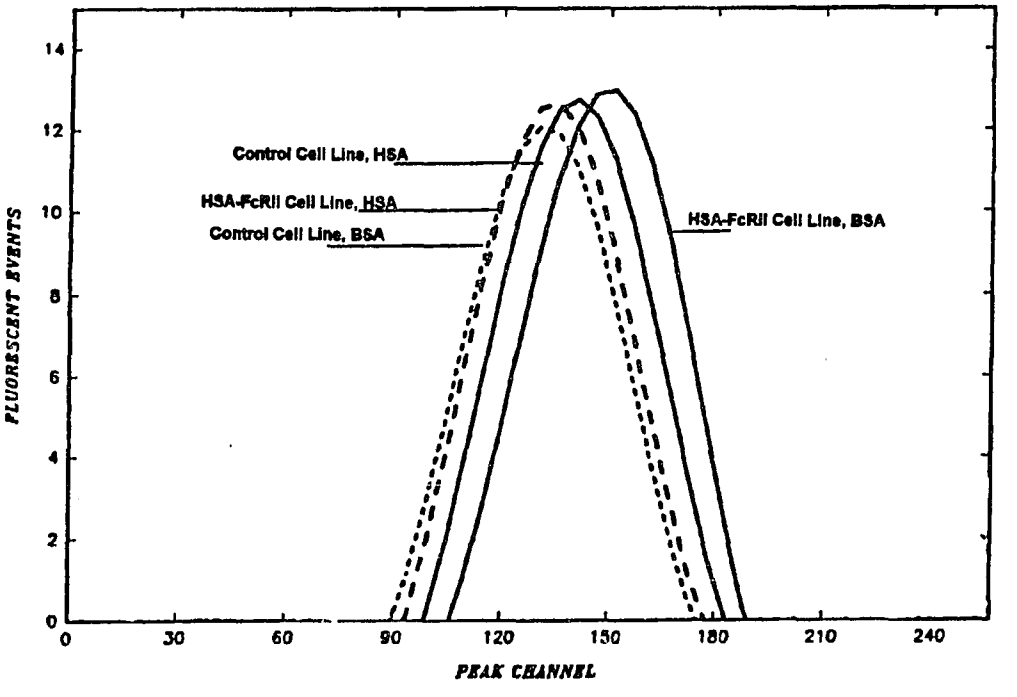
Figure 4 FACS analysis of HSA-FcγRII expression. **Top,** Expression of a chimeric HSA-FcγRII receptor. The HSA-Fc$_\gamma$RII and P-0 cell lines were stained with goat F(ab')$_2$ anti-HSA antibody which was followed by FITC-conjugated rabbit F(ab')$_2$ anti-goat IgG, as detailed in Materials and Methods. Samples were analyzed in a Coulter Epics Cell Sorter (FACS analysis) and the mean peak fluorescence channels (on a three decade log scale) are presented above. The staining of the HSA-Fc$_\gamma$RII cell line with the anti-HSA reagent is compared to the staining obtained with a negative isotype control antibody reagent, goat F(ab')$_2$ anti-IgD, as well as the negative staining of the P-0 line with either reagent. These data are tabulated on Table 2. **Bottom,** Competitive binding study. The staining of the HSA-FcγRII and P-0 cell lines with anti-HSA in the presence of 10 mg/ml BSA or HSA is shown. These inhibition data are tabulated under Experiment 1 on Table 2.

FACS Analysis of HSA-FcγRII Expression

Expression of Chimeric HSA-FcRII Receptor



Competitive Binding Study



literature of an albumin--like molecule, B700, which is expressed by murine melanoma cell lines (341,342). N-terminal amino acid sequencing suggests that B700 is in fact more homologous to BSA than to HSA or MSA (341), whereas the results presented in Figure 4 and in Table 2 show that HSA is the best competitor. Moreover, mAbs raised to B700 fail to crossreact with any albumins tested (including BSA and HSA) other than MSA (342). Control and HSA-FcRII cells were stained with anti-HSA antibody with the addition of various albumins at 10 mg/ml in the staining medium. The background staining seen with the control cell line is slightly increased in the first of two experiments shown in Table 4 and illustrated in Figure 4 and probably represents nonspecific absorption of the added albumin to the cell surface. The reason for this variable background in the presence of HSA is not clear. Nevertheless, the anti-HSA staining of the HSA-FcRII cells clearly remains positive above the background staining in the presence of BSA or MSA; only HSA can completely inhibit the positive staining. Staining was quantitatively compared by assigning values of 100% and 0% staining to the HSA-FcRII and P-0 cells that were stained with anti-HSA. Based on conversion of the differences in peak channel values to linear fold increases in fluorescence, the % positive staining was calculated for each of the inhibition conditions. The % inhibition of maximal positive staining was 6% for BSA, 34% for MSA, and highest, 99% for HSA. The results are displayed in Figure 4 and Table 2. These data strongly argue that the positive staining detected on the HSA-FcRII cell line by the anti-HSA antibody is indeed due to expression of the chimeric receptor.

To study the function of the chimeric HSA-FcRII receptor, we analyzed the surface expression of the receptor following crosslinking by FACS. The HSA-FcRII cell line was stained with $F(ab')_2$ goat anti-HSA antibody at 4°C, washed, and then incubated at 37°C for various times prior to staining with $F(ab')_2$ FITC-anti-goat IgG antibody. In this assay, a decrease in staining represents loss of the chimeric receptor from the cell surface. The results, presented in Figure 5 and Table 3, indicate that the crosslinked HSA-FcRII receptor is internalized rapidly (39% by 2 min, and 100% by 10 min) from the plasma membrane upon crosslinking. Moreover, the clearance is temperature dependent (compare middle and bottom panels of figure 5; see Table 3). While the clearance of the chimeric receptor is virtually complete following a 10 min incubation at 37°C, only a partial internalization of 45% was observed following a 10 min incubation at 16°C. (Compare the middle and bottom panels of Fig. 5 which show the staining before and following incubation at 37°C and 16°C, respectively, with the top panel, which shows the maximal and background staining levels.) This marked temperature dependence implicates a phagocytic rather than pinocytic mechanism of internalization (343). It should be noted that the differential effect of temperature on various endocytic processes is not an absolute differentiating criterion. These data do not rule out the possibility that the chimeric receptor is shed, rather than internalized, upon crosslinking. However, the rapidity, completeness, and temperature dependence of the clearance support an internalization mechanism. Moreover, in technically identical studies of P388D1 cells expressing the huFcγRIIA receptor (215), it was rigorously shown that the crosslinked receptor was internalized

Internalization of HSA-FcγRII upon Crosslinking

Experiment 1

Cell Line	Antibody	Temp	Time	Peak Channel	(Restained)	% Internalization
P-0	αIgD	4°	-	125		
"	αHSA	4°	-	131		100%
HSA-FcγRII	αIgD	4°		124		
"	αHSA	4°		151		0%
"	"	16°	30 min	143	(147)	45
"	"	37°	10 min	125		100
"	"	"	30 min	122	(129)	100

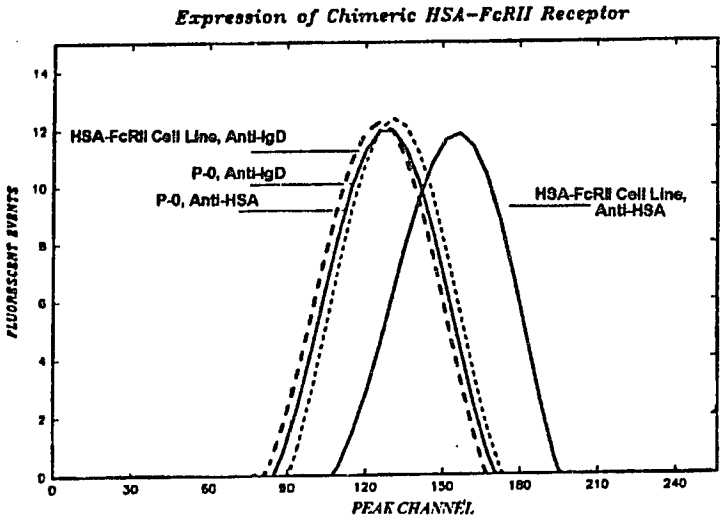
Experiment 2

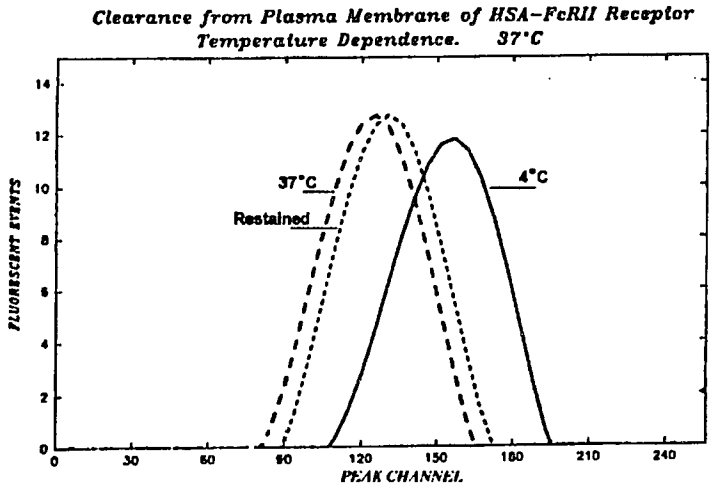
Cell Line	Antibody	Temp	Time	Peak Channel	(Restained)	% Internalization
P-0	αIgD	4°	-	100		
"	αHSA	4°	-	105		100%
HSA-FcγRII	αIgD	4°	-	99		
"	αHSA	4°	-	126		0
"	"	16°	10 min	121		28
"	"	37°	2 min	119		39
"	"	37°	10 min	99		100

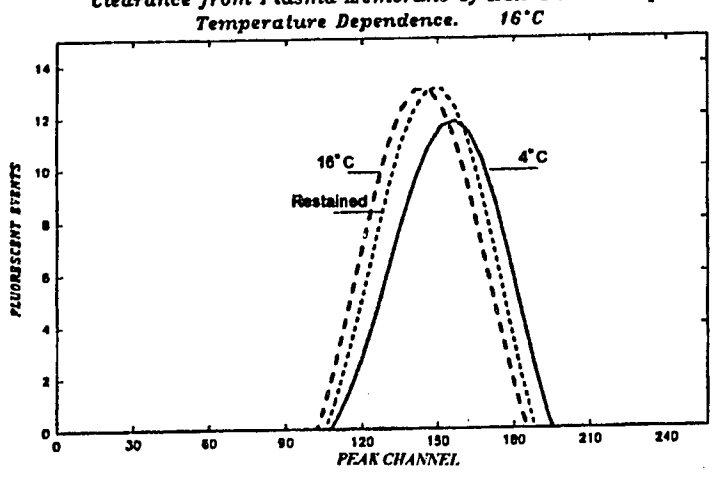
Table 3 Internalization of HSA-FcγRII upon crosslinking. The HSA-FcγRII cell line and the control cell line, P-0, were stained as detailed in Materials and Methods. Following incubation at 4°C with the crosslinking goat $F(ab')_2$ α-HSA polyclonal antibody, samples were washed. Some samples were then shifted to the indicated temperatures for variable times. Subsequently, the level of *remaining* surface receptor was assayed with a 2° FITC-conjugated rabbit α-goat IgG using a Coulter Epics Cell Sorter. Values obtained with the α-HSA reagent following the 4°C incubation for the HSA-FcγRII and P-0 cell lines were taken as 0% and 100% internalization values. Shifts in log fluorescence, relative to the background staining of P-0 with α-HSA, were converted to linear fold increase in fluorescence. The % of receptor internalized following incubation at 16°C or 37°C was calculated using the following equation: 1 - [(fold increase in fluorescence at 37°C - 1)] ÷ [(fold increase in fluorescence at 4°C - 1)].

Figure 5 FACS analysis of HSA-FcγRII internalization. **Top,** Maximal positive staining of the HSA-FcγRII cell line relative to negative antibody and cell line controls (as previously detailed in Fig. 4). All data presented herein were obtained in the same experimemt. **Middle and Bottom,** Temperature dependence of HSA-FcγRII clearance (internalization). In the clearance experiments, the HSA-FcγRII cell line was stained with the polyclonal anti-HSA antibody which crosslinks the receptor. Following washing, samples were either held at 4°C or transfered to 16°C or 37°C for 30 minutes. All samples were then stained with FITC-conjugated reagent at 4°C and analyzed on a Coulter Epics Cell Sorter. Data are tabulated under Experiment 1 in Table 3.

FACS Analysis of HSA-FcγRII Internalization







by demonstrating cellular retention of rhodamine- and ^{125}I- labeled crosslinking reagents following 37°C incubations.

P388D1 cells are not capable of a superoxide burst nor release of lysosomal hydrolases, even following IFN-γ treatment (our unpublished results). The P388D1 cell line is capable of a $[Ca^{2+}]_i$ flux in response to huFcγRIIA stimulation (215). Unfortunately, expression of the chimeric receptor by the HSA-FcRII cell line was lost before this and other experiments could be accomplished. The reason for the lost expression remains unclear, as even early freezings of the line did not demonstrate expression when grown up. A subtle difference in cell culture conditions which would affect the β-actin promoter governing the chimeric cDNA is believed responsible. Numerous and varied attempts to assess the contribution of FCS source, plating density and culture splitting frequency were unrewarding. Attempts to re-establish the line through new transfections also proved unsuccessful, suggesting the alternative explanation of nonoptimal commercial antibody reagents. New reagents were procured, but this did not alter the findings.

Discussion

In this work, I established a transfection protocol for a macrophage--like cell line and developed a quick and sensitive FACS assay to study the fate of crosslinked surface--expressed receptor. These techniques were subsequently employed to assess the structure-function relationships of the huFcγRIIA receptor by Dr. Joseph A. Odin (215) and by Ching-Tai Lin (335), both also of this laboratory.

My preliminary findings demonstrated that a chimeric FcγR which lacked the extracellular domain was capable of receptor--mediated internalization of soluble

immune complexes bound to the receptor. It seems likely that this *in vitro* response reflects the *in vivo* phagocytic response, as both processes are rapid, complete, and markedly temperature dependent.

These results also argue that the signalling for FcγR-mediated (specifically, the muFcγRIIb2-mediated) internalization of immune complexes does not proceed through any required conformational change in the extracellular domain. This mechanism stands in contrast to the signalling mechanisms of receptors for various hormones and neurotransmitters. When my studies were conducted, they were the first to directly address the role of the extracellular domain of FcγRs. My result was not unexpected, as a great wealth of data in the prior literature demonstrated the absolute requirement of receptor crosslinking for signalling (12,14-16,18,35). Subsequent recent work has successfully demonstrated that the extracellular domains of human FcγRII (A and C) are not required for the $[Ca^{2+}]_i$ flux and ADCC response when expressed in a T cell line and in primary monocytes (216).

It is now recognized that many FcγRs are members of the Multichain Immune Recognition Receptor (MIRR) family. Individual receptors share identical or closely related subunits which themselves are members of yet another family, the zeta family. The γ, ζ, and η chains are currently believed to be central in the signalling mechanisms of the MIRR receptors. The multisubunit structure of the MIRRs may explain why none of these receptors appears to be dependent on conformational signals generated through specific interactions of the natural ligand with the receptor; all of the MIRR receptors can be stimulated nonphysiologically with various anti-receptor crosslinking antibodies. As suggested by Keegan and Paul (27) in their

elegant article, the evolution of multisubunit receptors may have enabled the receptors of the immune system to signal in response to a vast variety of antigens which crosslink the receptors in a myriad of orientations.

Methods

Construction of a chimeric HSA-muFcγRIIb2 expression vector

I designed a chimeric HSA-muFcγRIIb2 construct which encodes 583 of the 585 amino acids of human serum albumin (HSA) linked to the transmembrane and cytoplasmic domains of muFcγRIIb2. The construct was initially made in puc12 and then cloned into the LK444 expression vector (344). The LK444 vector provides the β-actin promoter and a neomycin resistance gene. I used a plasmid which would contain both the cDNA of interest as well as a selectable marker to increase the percentage of G418-selected colonies which would express the gene of interest versus that of a cotransformation with two separate plasmids. All ligations were performed in low melting point agarose (345) with T4 ligase and were transformed into the LM1035 strain of HB101. Linkers were phosphorylated with T4 kinase (345).

The cDNA encoding HSA was isolated as a 2.0 kb BamH I-Sal I fragment from the pLJ-HSA retroviral vector (346) was graciously given by Dr. Richard Mulligan. This fragment was ligated into puc12 which had been linearized with BamH I and Sal I and dephosphorylated with calf intestinal alkaline phosphatase (CIP) (345). An Mlu I 8-mer linker was inserted at the Bsu36 I site [blunt ended with Klenow fragment of DNA polymerase (345) which followed the codon for amino acid 583 in the HSA cDNA. This construct is termed puc-HSA-mlu.

The ~600 bp Apa I-Pst I fragment of the muFcγRIIb2 cDNA encodes the 7 membrane--proximal amino acids of the extracellular domain and the transmembrane and cytoplasmic domains; it also contains 3' untranslated sequence. Following blunt ending with T4 DNA polymerase (345) this fragment was also linked to the Mlu I 8-mer. Following digestion with Mlu I to remove excess linkers, the fragment was isolated from a low melting point agarose gel and ligated into the CIP-treated Mlu I site of puc-HSA-mlu. Correct orientation of the inserted fragment was determined by Hinc II digestion of plasmid DNA isolated by the rapid boiling method (345). The resultant plasmid is termed puc-HSA-mlu-FcR. Inclusion of the linker was necessary to maintain the correct reading frame of the muFcγRIIb2 cDNA.

The 2.6 kb chimeric cDNA was isolated as follows: puc-HSA-mlu-FcR was first digested with Sca I and Hind III and blunt ended with Klenow fragment, and BamH I linkers were ligated to these ends. Following digestion with BamH I, the 2.6 kb cDNA was isolated and cloned into the CIP-treated BamH I site of LK444. Correct orientation, relative to the β-actin promoter, was determined by Mlu I-EcoR I digestion. The resultant chimeric expression vector is termed LK-HSA-muFcγRIIb2. Plasmid DNA was isolated by a modified alkaline lysis method and CsCl density equilibrium centrifugation (345).

Transfection of P388D1 with LK-HSA-muFcγRIIb2

My goal was to express the chimeric HSA-muFcγRIIb2 cDNA in a cell type capable of normal macrophage functions. I attempted to transfect three murine macrophage--like lines, J774, Raw264.7, and P388D1, and succeeded with the P388D1 line. The $CaPO_4$-DNA coprecipitate was prepared as follows. For

transfection of cells on a 100 mm dish, 2 ug of LK-HSA-muFcγRIIb2 plasmid and 18 ug of carrier salmon sperm DNA was added to 0.5 ml of 0.25 M $CaCl_2$. This solution was added dropwise to 0.5 ml of 2x Hepes buffered saline (2x HBS = 280 mM NaCl, 50 mM Hepes, cell culture grade, and 1.5 mM sodium phosphate, pH 7.1 +/- 0.05). During the dropwise addition, the solution was continuously agitated with a stream of air bubbled in through a pipet. The mixed solution was allowed to stand at room temperature for 30 minutes.

P388D1 cells are maintained in DMEM with 5% FCS. For transfection, the cells were plated at 5-8 x 10^5 cells per 100 mm dish and cultured overnight. On day 1, plates were rinsed and fed with 10 ml of transfection medium: DMEM, 5% FCS, 100 uM chloroquine. One ml per plate of the $CaPO_4$-DNA coprecipitate was added dropwise and plates were swirled to mix. Plates were incubated for 4-8 hours in a 37°C, 5% CO_2 incubator. Following this incubation, plates were rinsed and fed with regular medium (DMEM, 5% FCS).

On day 2, plates were rinsed and fed with selective medium: DMEM, 5% FCS, 0.2 mg/ml G418. Selective medium was replaced every 3-4 days. Colonies of resistant cells were visible at 2-3 weeks and were cloned with metal cylinders at 4 weeks post transfection and maintained in selective medium. Approximately 20-50 colonies were obtained per plate. No resistant colonies were ever seen in mock transfection control plates. Chloroquine was essential for successful transfection. It is believed that chloroquine may inhibit lysosomal degradation of the DNA; the uncharged base partitions into the lysosomal compartment and neutralizes the pH (345).

PCR sequencing of the transfected HSA-muFcγRIIb2 DNA

The sequence of the chimeric HSA-muFcγRIIb2 chimeric construct was confirmed from ~100 bp. (in the HSA sequence) (346) 5' to the linker junction, through the linker junction, and through the entire coding sequence and some 3'UT sequence of the muFcγRIIb2 (88) portion of the construct. To do this, genomic DNA was isolated from the transfected cell line which was shown by FACS analysis to express HSA. Frozen nuclei pellets obtained from 0.65% NP40 cell lysates were treated with 0.5% SDS and 100 ug/ml Proteinase K. Nucleic acid was extracted with phenol/chloroform and ethanol precipitated (347). Two 17-mer oligonucleotides--one from HSA, 5' to the linker junction, and the other from the 3' UT region of muFcγRIIb2--were used to PCR-amplify the expected 600 bp. fragment unique to the transfected cell line (348). The oligonucleotides were purified by passage over a SEP-PAK C-18 column (Waters). The 10x PCR buffer is 500 mM KCl, 100 mM Tris-HCl, pH 8.3 at room temperature, 1.5 mM MgCl, 0.1% w/v gelatin, and 1% Triton X. The optimized PCR conditions were: denaturation, 1 minute, 94°C, annealing, 72°C, 2 min, and extension, 47°C, 3 minutes. Specificity of the reaction was monitored by comparison with the PCR reaction of DNA from the control cell line transfected only with the original LK444 vector. The 600 bp. fragment of 10 pooled PCR reactions, each amplifying 500 ng of genomic DNA, was isolated from low melting point agarose gel by hot phenol extraction. This 250 ng of DNA PCR product was again amplified by PCR, using 50 ng per PCR reaction. This product was treated with RNase, extracted with phenol/chloroform, and precipitated with isopropanol/NH_3OAc to yield 1800 ng of DNA. This twice amplified DNA was

sequenced (using 280 ng) with Sequenase 2.0 (U.S. Biochemical Corp.), according to the manufacture's instructions, except that denaturation was accomplished by boiling for six minutes followed by 1 minute at room temperature. The sequencing result (data not shown) demonstrated that the linker junction of the chimeric construct is in frame and furthermore showed that no mutations have occurred in the integrated DNA of the chimeric HSA-muFcγRIIb2 construct.

FACS analysis of the chimeric HSA-muFcγRIIb2 expressed in P388D1

Transfected cell lines were screened for expression of the chimeric receptor by FACS using an F(ab')$_2$ goat anti-HSA polyclonal antibody and a FITC F(ab')$_2$ rabbit anti-goat IgG polyclonal antibody (Organon Technica). Transfectants obtained using only the original vector, LK444, served as a negative control cell line (P-0). Of 6 lines screened, 3 positives were identified. As these three lines had all arisen in the same original tissue culture plate, it was assumed that they may in fact only represent one true original transfectant, and thus one of the three, termed HSA-FcRII, was chosen for further study. The background staining of the HSA-FcRII line and of the P-0 control cell line with an irrelevant control antibody, a polyclonal F(ab')$_2$ goat anti-IgD, were identical. Control cells and HSA-FcRII cells were collected and washed once in staining medium (DMEM, 2% chicken serum, 20 mM Hepes, pH 7.4), and resuspended at 1 x 10^6 cells in 0.1 ml of staining medium containing either anti-HSA or anti-IgD antibody. Incubation was for 1 hour on ice. Following two washes at 4°C, cells were stained under the same conditions with the secondary FITC-F(ab')$_2$ rabbit anti-goat IgG. Following washing, cells were fixed in 3.7% formalin on ice and analyzed by FACS. To study the fate of the chimeric

receptor following crosslinking, the staining procedure just detailed was altered as follows: The HSA-FcRII cell line was stained with $F(ab')_2$ goat anti-HSA antibody at 4°C, washed, and then incubated at 37°C (or at 16°C) for various times. Incubations at 37°C (or at 16°C) were ended by addition of a large volume (1 ml) of cold (4°C) staining medium. Cell pellets were collected and stained at 4°C with $F(ab')_2$ FITC-anti-goat IgG antibody, and then washed and fixed for FACS analysis as detailed above. In this assay, a decrease in staining represents loss of the chimeric receptor from the cell surface.

III FcγRIIA SIGNALLING: TYROSINE PHOSPHORYLATION

Introduction

Receptors for the Fc domain of IgG (FcγR) on leukocytes mediate a pleiotropic response following crosslinking by immune complexes. The details of signal transduction after FcγR crosslinking are not well understood. These receptors are not coupled to G proteins nor do they have intrinsic enzymatic activity. Recent observations suggest that signalling events following crosslinking of B and T cell antigen receptors, $Fc_\epsilon RI$, and FcγRs, share common elements (26,27). In each, signalling is initiated by receptor crosslinking by antigen or immune complexes. Subsequent events include phosphorylation on tyrosine (111,121,128,260,266), activation of phospholipase C-γ 1 (298), and $[Ca^{2+}]_i$ flux (190). There is a conserved tyrosine activation motif with a dyad Y-X-X-L sequence with the two tyrosines separated by 10 amino acids, which is present in Igα, Igβ, FcεRIγ, and CD3ε and ζ (Figs. 2 and 3). Site-directed mutagenesis studies of CD3ζ suggest that the two tyrosines in the conserved motif are crucial (30). A related motif, in which the Y-X-X-L sequences are separated by an additional 5 residues, is found in FcγRIIA (Fig. 3).

Human FcγRIIA found on macrophages and neutrophils has two well documented functions -- to mediate the phagocytosis of immune complexes and to trigger the $[Ca^{2+}]_i$ flux required for the oxidative burst (349). Previously, the structural requirements for these activities were analyzed by expressing FcγRIIA and truncated FcγRIIA proteins in murine macrophage (P388D1) and fibroblast (CHO) cell lines (215). In these studies, human FcγRIIA was specifically crosslinked using

mAb IV.3 Fab (anti-human FcγRII) and an anti-mouse IgG F(ab')$_2$ reagent. These studies demonstrated that CHO cells lack the signalling apparatus required for either FcγRIIA-mediated phagocytosis or $[Ca^{2+}]_i$ flux and identified a 31 residue cytoplasmic region (Arg^{234}-Asp^{264}) required for phagocytosis in P388D1 cells. Surprisingly, deletion of the carboxyl-terminal 17 residues, Lys^{265}-Asp^{281} (Δ264), which included the distal Y-X-X-L motif, ablated the $[Ca^{2+}]_i$ flux while preserving the ability of the receptor to mediate phagocytosis of complexes in P388D1 cells.

Other studies of FcγR transfectants in T cell and fibroblast cell lines have also suggested that the cytoplasmic domains are important in signal transduction (28,58,125,128,216,217,234,235,350,351). The macrophage-based FcγRIIA expression system we have used permits a more physiologically relevant analysis of structure-function relationships and signal transduction than some other systems such as COS cells (58,128) and baby hamster kidney cells (BHK-21) (217), as FcγRs are normally functional in P388D1 cells. Site--directed mutagenesis studies were undertaken by Ching-Tai Lin (335) to assess the importance of specific residues between Arg^{234}-Asp^{264} for signal transduction (see Fig. 6 for location of mutations), and his results concerning phagocytosis and $[Ca^{2+}]_i$ flux are summarized in Table 4. I analyzed the role of tyrosine phosphorylation in FcγRIIA signalling and compared tyrosine phosphorylation events following crosslinking of wild type (wt) and mutant FcγRIIA expressed in the P388D1 mouse macrophage--like cell line (335).

My hypothesis of huFcγRIIA signalling was based on the following observations: huFcγRIIA and other MIRRs are activated similarly by receptor crosslinking and they share closely related tyrosine activation motifs. Moreover, this

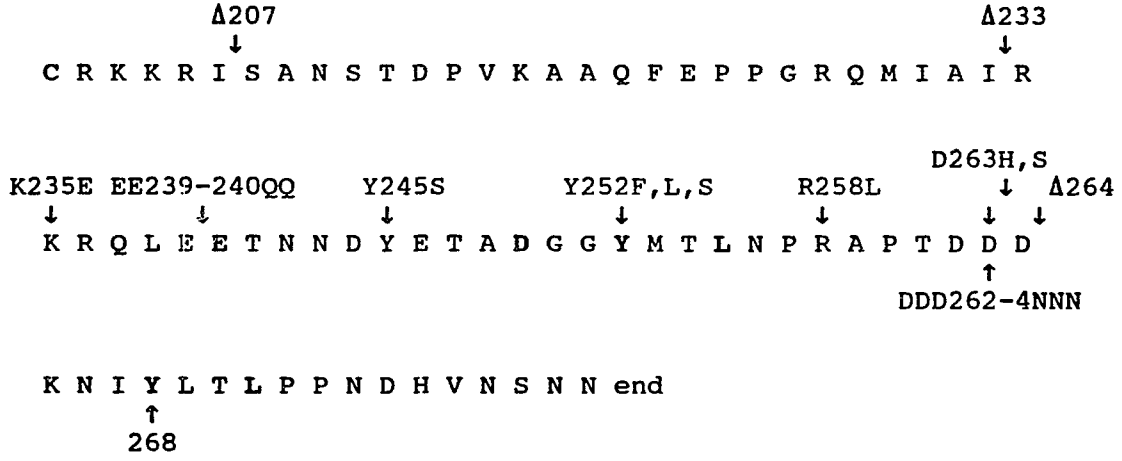


Figure 6 Location of deletion and point mutations (constructed by Dr. Joseph A. Odin and Ching-Tai Lin, respectively) in the cytoplasmic domain of the huFcγRIIA (215,335). The sequence of the wildtype FcγRIIA is shown in the single-letter amino acid code, with residues of the MIRR motif in bold. Three truncated molecules, Δ207, Δ233, and Δ264 which retain 4, 30, and 74 amino acids (and are lacking the carboxy-terminal 74, 48, and 17 residues) respectively, are indicated. The point mutants are notated with the wildtype residue, numeric position, and mutant residue. Thus, for example, the lysine at position 235 has been changed to glutamate in the K235E mutant.

	WT	264	233	207	K235E	EE239-240QQ	Y245S	Y252F	Y252L	Y252S	R258L	D263H	D263S	DDD263-264NNN
Internal.	+	+	-	-	+	+	+	-	+	+	+	+	+	+
Phago.	+	-	-	-	+	+	+	-	+	+	+	+	+	+
$[Ca^{2+}]_i$	+	-	-	-	+	+	+	-	+	+	+	+	+	+
Y Phosphor.	+++	+	-	-	+++	+++	+++	++	+++	+++	+++	+++	+++	+++

Table 4 Summary of the functional capacities of wildtype (wt) and deletion and single point mutant $Fc_\gamma RIIA$. The deletion mutants were constructed and analyzed (excepting for Y phosphorylation) by Dr. Joseph A. Odin (215). The single point mutants were constructed and analyzed (excepting for Y phosphorylation) by Ching-Tai Lin (335). The Δ264, Δ233, and Δ207 mutants lack 17, 48, and 74 carboxy-terminal residues and retain cytoplasmic tails of 61, 30, and 4 amino acids, respectively. The point mutations are noted using the single letter amino acid code. Thus, for example, lysine at position 235 has been changed to glutamate in the K235E mutant. Internal.= receptor internalization in response to crosslinking using anti-receptor antibodies and a secondary crosslinking antibody. Phago.= phagocytosis of erythrocytes opsonized with anti-receptor antibodies. $[Ca^{2+}]_i$ = mobilization of calcium in response to receptor crosslinking. Phosphor.= tyrosine phosphorylation of multiple cellular targets induced upon receptor crosslinking.

motif has been shown to be critical for TCR signalling. Additionally, activation of tyrosine kinases has been demonstrated to be the earliest known signal transduction event for other MIRRs, apparently preceding the $[Ca^{2+}]_i$ flux as well as other cellular effector functions. Thus I proposed that huFcγRIIA signalling would involve signal transduction mechanisms analogous to other MIRRs. Specifically, huFcγRIIA signalling would rapidly activate tyrosine phosphorylation/dephosphorylation cascades that would precede other receptor--mediated functions including a $[Ca^{2+}]_i$ flux and phagocytosis.

This proposed order of the signal transduction would be assessed by two methods: 1) Pharmacological inhibitors of tyrosine kinases (herbimycin A) and of $[Ca^{2+}]_i$ flux (BAPTA-AM) would be used to analyze the dependence of each response upon the other. 2) Analysis of point mutants may allow the dissection of various signalling pathways. It was expected that some mutants, for example, might retain the tyrosine phosphorylation response while one or more proposed downstream responses, such as a $[Ca^{2+}]_i$ flux and phagocytosis, would be abrogated. Secondly, the tyrosine activation motif would be required for huFcγRIIA signalling, demonstrated though the study of deletion and point mutants.

Results

Protein Tyrosine Phosphorylation Upon Crosslinking FcγRIIA. The kinetics of protein tyrosine phosphorylation in response to crosslinking the wt FcγRIIA were examined. As was done in the $[Ca^{2+}]_i$ flux experiments (215,335), the cells were precoated with mAb IV.3 Fab and were then triggered by crosslinking with the anti-mouse IgG $F(ab')_2$ at high concentration (40 μg/ml). This enabled synchronous

stimulation of the huFcγRIIA. Multiple bands of increased intensity were detected in post-nuclear detergent lysates by immunoblot analysis using the anti-phosphotyrosine mAb 4G10 (Fig. 7). Stimulations were ended by immediate transfer of the cell suspension (100 μl) into a larger (1.2 ml) volume of PBS- 2% FCS which had been adequately prechilled (> 30 min) in a salt-ice water bath (approximately *minus* 3°C). Attention to these aspects proved to be absolutely critical for experimental success. Additionally, optimal signal to noise ratios for the Western blot analysis were obtained with the BSA Tween-20 (0.05%) blocking solution. Alternate blocking solutions employing stronger detergents and/or non-fat dried milk resulted in a substantially decreased signal.

The apparent molecular weights (M_r) of bands in the groups indicated in Figure 7 are listed in Table 5. Most bands exhibiting increased tyrosine phosphorylation are apparent by 30 sec, although several are not evident until 1 min. Close inspection of the original blots revealed 22 bands exhibiting increased intensity following FcγRIIA stimulation, with 9-10 of these bands exhibiting increases in intensity which are easily appreciated in the scan of the blot presented in Figure 7. Generally, phosphorylation was transient with maximal intensity between 1-2 min and returned to baseline levels by 5-10 min. A major band at 74,000 M_r was visible as early as 15 sec after crosslinking. In contrast to the induction of the 69,500 M_r band seen in some experiments (see Fig. 7, panel A), in other experiments the 69,500 M_r band was visualized at time 0 (compare wt time 0 lanes of Fig. 7, panels A, B, and C). The reason for this finding is not known. By 10 min nearly

Figure 7 Time course of protein tyrosine phosphorylation following FcγRIIA crosslinking. **A,** WT huFcγRIIA expressed in the P388D1 macrophage--like cell line; **B,** Δ264 truncation; **C,** Y252F point mutation. FcγRII were labeled at 37°C with the Fab mAb IV.3, washed and then crosslinked with goat anti-mouse IgG $F(ab')_2$ for the indicated times prior to stopping the stimulation by transfering the cell suspension to large volume of prechilled PBS-2% FCS. Post-nuclear lysates were electrophoresed and blotted with anti-phosphotyrosine. Each lane represents 3 x 10^5 cell equivalents. The six groupings of bands exhibiting increased anti-phosphotyrosine reactivity after stimulation are indicated and are tabulated according to apparent molecular weight and relative intensity in Table 5. The earliest apparent band of 74,000 Da is indicated by the arrow in the 15s lane of panel A. In panel B, arrows indicate the groups of missing bands. The 1* designation means that only the higher M_r bands of 140,000 and 142,000 Da are missing from group 1. The 3* denotes that only the upper band of 85,000 Da of group 3 is missing in panels B and C.

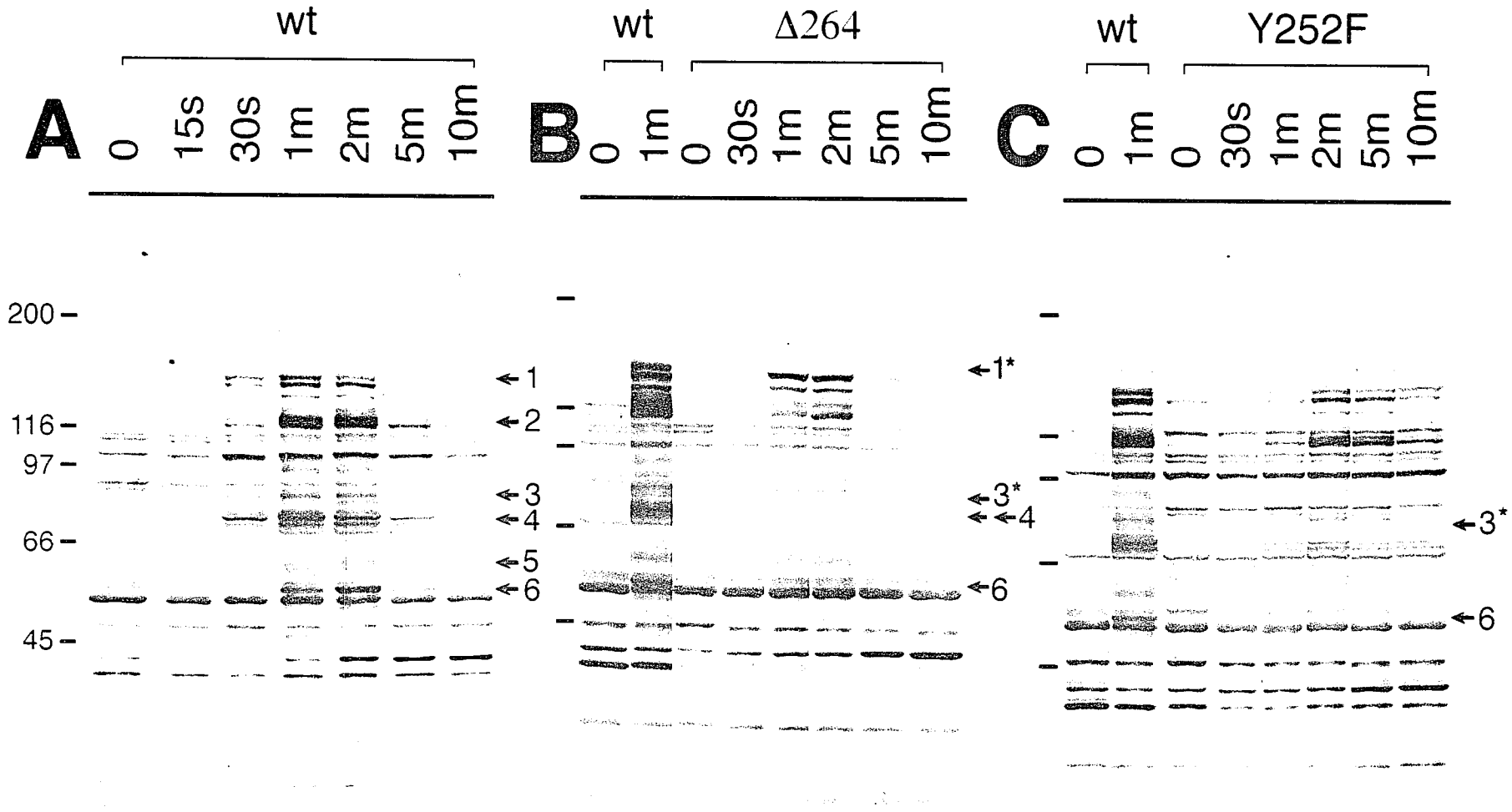

A
wt
0
15s
30s
1m
2m
5m
10m
B
wt
Δ264
0
1m
0
30s
1m
2m
5m
10m
C
wt
Y252F
0
1m
0
30s
1m
2m
5m
10m
200
116
97
66
45
1
2
3
4
5
6
1*
3*
4
6
3*
6

Figure 7

M_r of Y-Ⓟ bands	group	wild-type	Δ264	Y252F	PMA	Herb A
155		+	+	+	nl	-
142	1	+	-	+	nl	-
140		+++	-	++	nl	-
137		+++	+++	+++	nl	-
130		+	+	+	nl	-
126		+	++	+	nl	-
119	2	++++	+++	++	nl	-
116.5		++++	+++	+++	nl	-
103	3	+++	+	+	nl	-
96.5		+	+	+	nl	-
85		++	+	++	↑ basal ↑ with stim.	-
82.5		+	-	-	↓ or absent	-
79.5	4	++	-	+	nl	-
74		++++	-	++	nl	-
72.5		++	-	++	nl	-
69.5		+	-	+	nl	-
62.5	5	+	+	+	nl	-
61		+	+	+	nl	-
58.5		+	+	+	nl	-
57.5		+	+	+	nl	-
51	6	++++	-	-	↓ induc-tion	present basally
49.5		++	-	-	nl	? nl

Table 5 Differences in protein tyrosine phosphorylation patterns upon activation of wt and mutant FcγRIIA. Relative intensities of the bands are indicated. Differences in response to stimulation of the wildtype receptor following pretreatment with PMA or Herbimycin A are also indicated. M_r, indicated in kDa, was calculated from migration distance on SDS-PAGE compared to that of MW standards. Y-Ⓟ, tyrosine phosphate.

all of the proteins phosphorylated on tyrosine in response to FcγRIIA crosslinking were no longer visible.

Two results support the interpretation that the increased intensity of phosphotyrosine--containing bands were due to *in vivo* responses to receptor stimulation which did not occur or continue *in vitro* following cell lysis. Firstly, a precise rapid and reproducible time course of phosphorylation/*dephosphorylation* was observed. This time course was reproducible moreover, in the face of substantially different *post*-lysis sample handling times prior to gel electrophoresis between various experiments. Secondly, the phosphorylation pattern observed was not altered by the absence or inclusion of EDTA in the lysis buffer. Rigorous demonstration of the absence of tyrosine kinase activity in the lysates by inclusion of γ-^{32}P-labeled ATP in the lysis buffer has not been done.

No stimulation of tyrosine phosphorylation (at 1 min) was observed in the control P388D1 cell line, PC1, that does not express huFcγRIIA. Additionally, no induction of tyrosine phosphorylation was detected at 1 min in a CHO cell line expressing wt FcγRIIA (Fig. 8). Moreover, mock stimulations (no mAb IV.3 Fab, only secondary crosslinking antibody) done in all experiments at each time point showed no stimulation other than a 37,000 M_r band. This protein cannot be FcγRIIA since it is also present in the PC1 control cell line. Likewise, treatment of the cells with mAb IV.3 Fab alone did not result in stimulation at any time point, again excepting the 37,000 M_r band.

Protein Tyrosine Phosphorylation in FcγRIIA Mutants. The protein tyrosine phosphorylation patterns stimulated by crosslinking all point and truncation mutants

Figure 8 Tyrosine pgosphorylation response of CHO, P-0, Δ233, and Δ207 cell lines. The responses to mAb IV.3 crosslinking of huFcγRIIA--negative cells lines, CHO and P-0, and the nonfunctional severe deletion mutants, Δ233 and Δ207, were analyzed by Western blotting. CHO is a fibroblast cell line; P-0 is derived from the same murine macrophage--like cell line, P388D1, as WT and the huFcγRIIA deletion and point mutants. Lysates from cells stained with the anti-huFcγRII mAb, IV.3, were stimulated for 1 min with goat anti-mouse IgG $F(ab')_2$ and then were electrophoresed and blotted with the anti-phosphotyrosine mAb 4G10. The six band groupings are indicated by arrows, as in Figure 7 and Table 5.

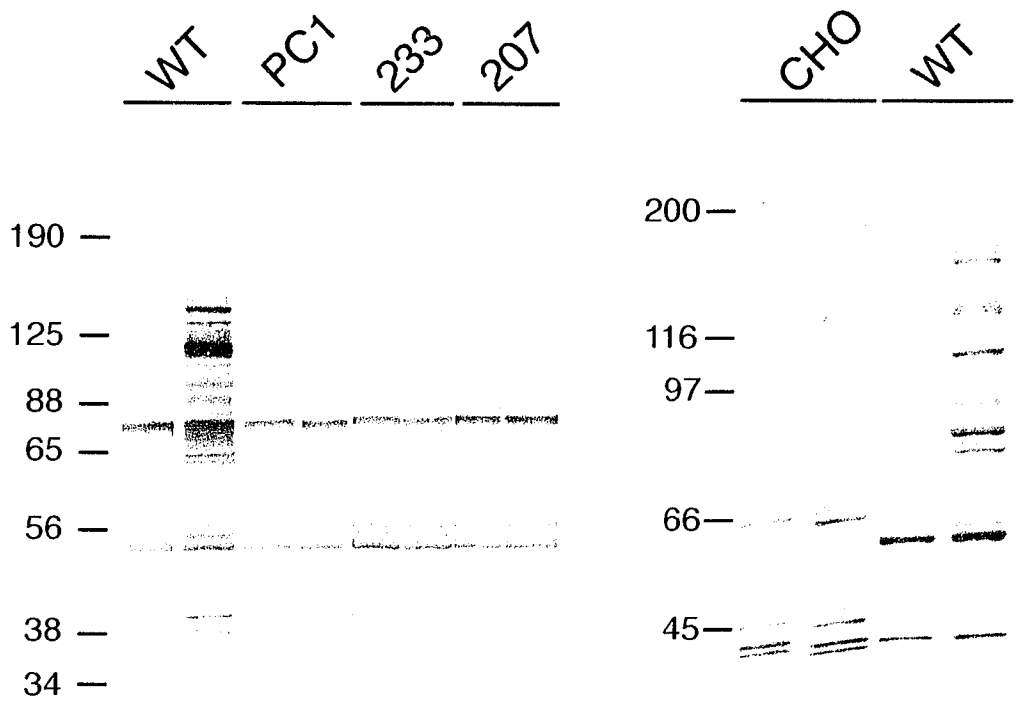


Figure 8

were examined. The nine point mutants with wt function for phagocytosis and $[Ca^{2+}]_i$ flux exhibited essentially wt tyrosine phosphorylation patterns and kinetics (Fig. 9, Table 4), whereas no induction was seen for two nonfunctional truncations, Δ233 and Δ207 (215) (Fig. 8, Table 4). The Δ264 truncation mutant (which cannot flux $[Ca^{2+}]_i$) lacked nine major phosphotyrosine-containing proteins of M_r 142,000, 140,000, 82,500, 79,500, 74,000, 72,500, 69,500, 51,000 and 49,500 (Fig. 7B; Table 5) that are seen in cells transfected with wt FcγRIIA. The kinetics of the Δ264 phosphorylation response were normal, however, with maximal intensities reached by 1-2 min and dephosphorylation occurring by 5-10 min. Surprisingly, the functionally inactive Y252F point mutant clearly lacked only three phosphotyrosine--containing bands of 82,500, 51,000, and 49,500 M_r, although the relative intensities of others, such as the 74,000 M_r protein were substantially less (Fig. 7C; Table 5). Notably, the phosphorylation response of Y252F was somewhat delayed and the dephosphorylation was significantly prolonged, with only 50% dephosphorylation of the M_r 140,000 protein seen after 10 min (Fig. 10).

Nine of 10 mutants, including Y252L, Y252S, and Y245S, presented essentially a wt phenotype for $[Ca^{2+}]_i$ flux, phagocytosis, and phosphorylation on tyrosine residues (Fig. 9, Table 4). The phenotype of the Y252S and Y252L mutants, which fluxed Ca^{2+} and phagocytosed E-IV.3 Fab as well as or better than wt, was a somewhat surprising result, since the Y252F mutant was nonfunctional for $[Ca^{2+}]_i$ flux and for phagocytosis of complexes as well as erythrocytes. Therefore, Ching-Tai Lin confirmed the presence of the mutations by sequencing the genomic

Figure 9 Comparison of Y245S, Y252S, Y252L, and Y252F cell lines. Lysates from cells stimulated by receptor crosslinking for 1 min (Y245S, Y252S, Y252L) or for 2 min (Y252F) were electrophoresed and blotted with 4G10 mAb. These time points were chosen for comparison as they are the time of maximal tyrosine phosphorylation response for each receptor. The six band groupings are indicated by arrows, as in Figure 7 and Table 5.

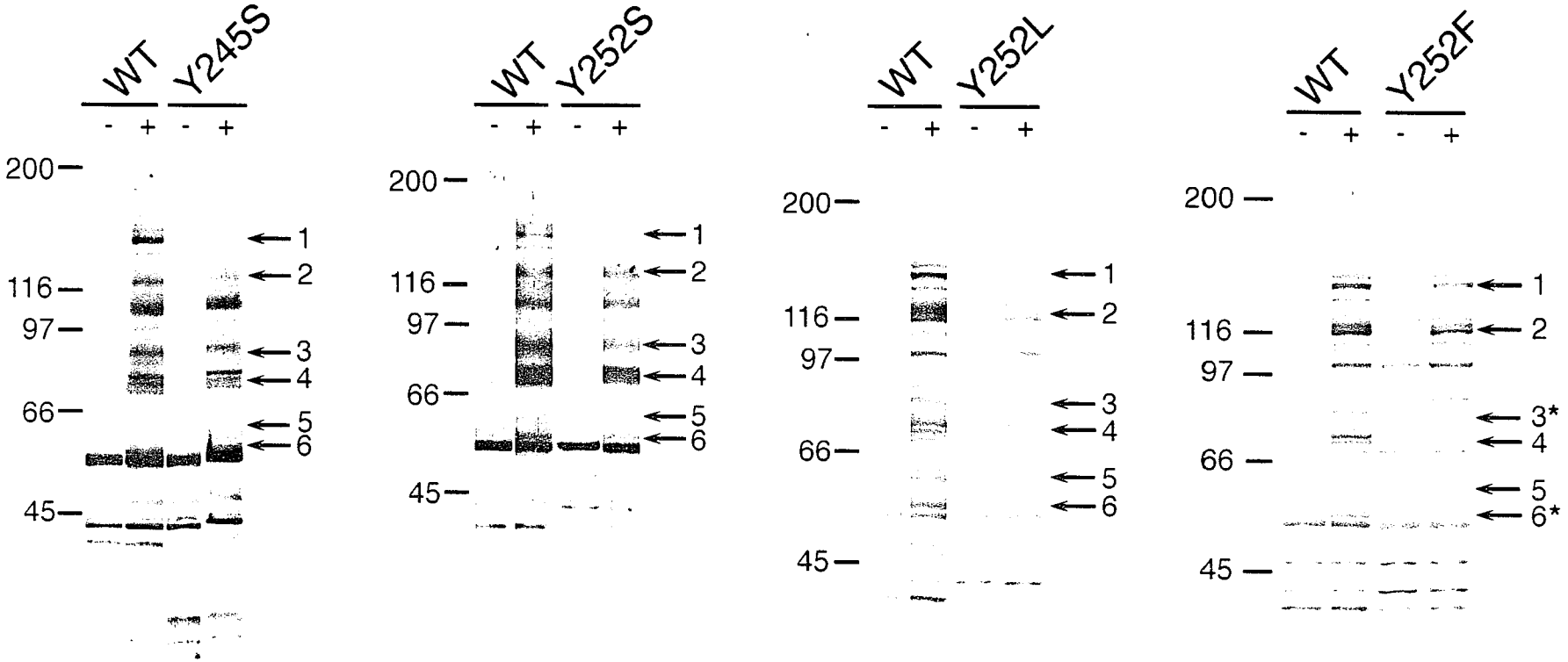


Figure 9

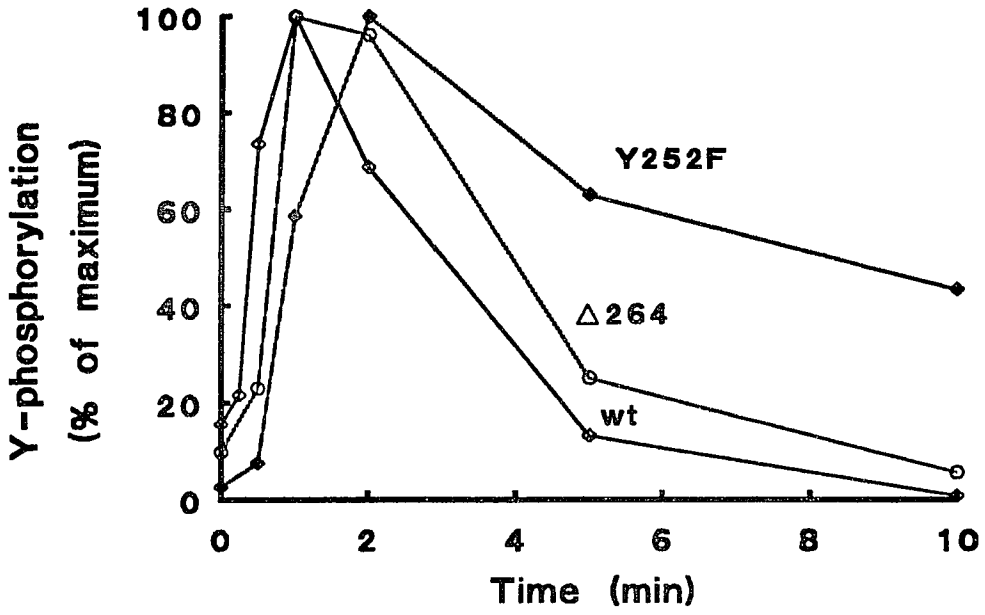


Figure 10 Quantitative comparison of the tyrosine phosphorylation response of wt, Δ264, and Y252F receptors. The intensity of the 140,000 Mr band of wt and Y252F and of the 137,000 Mr band of Δ264 were each divided by the intensity of the 45,000 Mr band constitutively present following stimulation of each receptor. This normalization was done in order to facilitate comparison of the three different receptor response kinetics. The 140,000 and 137,000 Mr bands were chosen as representative bands as they were each significantly well isolated for their densities to be precisely determined. (The 140,000 Mr band is not present in Δ264). The maximum response for each band was defined as 100%.

DNA of these transfectants after PCR amplification with appropriate primers (personal communication).

Pharmacological Studies of FcγRIIA Signalling. To assess the functional importance of protein tyrosine phosphorylation, P388D1 cells expressing wt FcγRIIA (PW16) were pretreated (10 μM, 20 hr) with the tyrosine kinase inhibitor, herbimycin A (260). This abrogated tyrosine phosphorylation in response to FcγRIIA crosslinking and reduced basal levels of tyrosine phosphorylated proteins (Fig. 10). The almost total lack of staining by mAb 4G10 of lysates from herbimycin-A treated cells also argues that the anti-phosphotyrosine staining we observe is specific. Both $[Ca^{2+}]_i$ flux and receptor-mediated phagocytosis of complexes were shown by Ching-Tai Lin to be inhibited in a dose-dependent fashion by herbimycin A pretreatment, with complete inhibition occurring at 10 μM herbimycin A (335). Loading P388D1 cells with BAPTA-AM (352), a calcium chelator, had no effect on phagocytosis of immune complexes (215,335) nor could I demonstrate any effect on protein tyrosine phosphorylation at 1 min (Fig. 11).

Protein kinase C is activated in the $Fc_\epsilon RI$ system following the receptor--mediated stimulation of phospholipase C (PI-PLC) with the resultant production of IP_3 and DAG, and the release of intracellular stores of Ca^{2+}. The potential involvement of PKC in huFcγRIIA signalling was examined by pretreatment of the wt cells with PMA, a pharmacological activator of PKC. Pretreatment for either 5 or 30 min resulted in increased basal intensity of the 85,000 M_r band and receptor crosslinking resulted in further increased intensity of this band. Additionally, PMA

Figure 11 Pharmacological studies of huFcγRIIA signalling. PMA (100 μM, 5 and 30 min), Herbimycin A (10 μM, 20 hr), and BAPTA-AM (100 μM, 30 min) pretreatments were followed by huFcγRIIA activation and the protein tyrosine phosphorylation response was analyzed by Western blotting with the anti-phosphotyrosine mAb 4G10. The six major band groupings are tabulated in Table 5. The 3* designation means that the upper 85,000 Da band of group 3 is increased in basal as well as stimulated phosphotyrosine content. In contrast, little if any induction of the lower 82,500 Da band of this group is apparent.

Figure 12 Immunoprecipitation of huFcγRIIA. An aliquot of the total wt lysate prior to immunoprecipitation was run in the first two lanes. MAb IV.3--immunoprecipitated receptor obtained following activation (by receptor crosslinking) for various times from wt cells was electrophoresed and blotted with the anti-phosphotyrosine mAb 4G10 (αPY). This time course (0-5min) is shown in lanes 3-7. Immunoprecipitates from huFcγRIIA--expressing (WT) and --nonexpressing (P-0) cell line lysates that were electrophoresed and blotted with a rabbit polyclonal anti-huFcγRIIA (RαFcγRIIA) reagent are shown in the last two lanes.

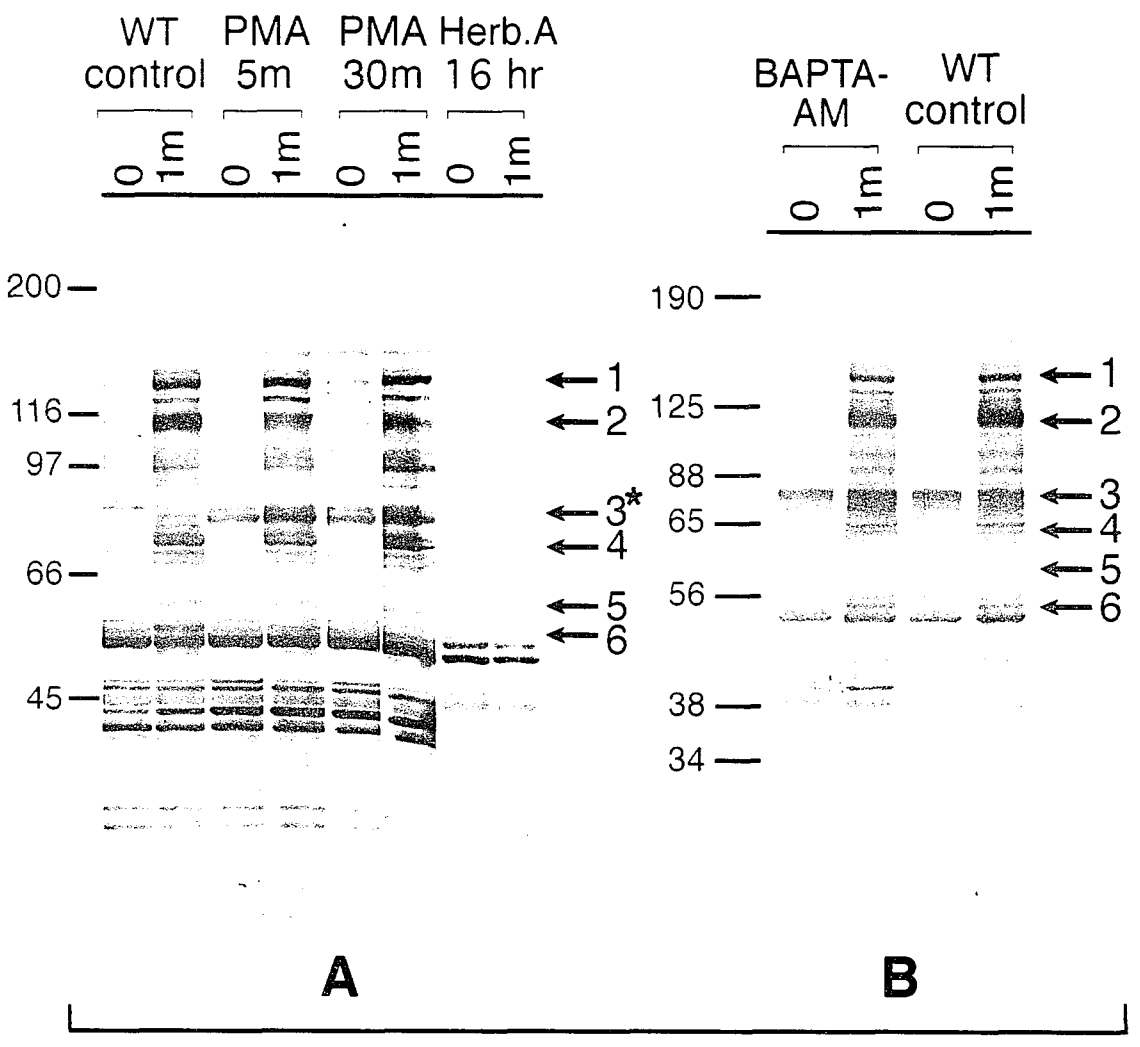


Figure 11

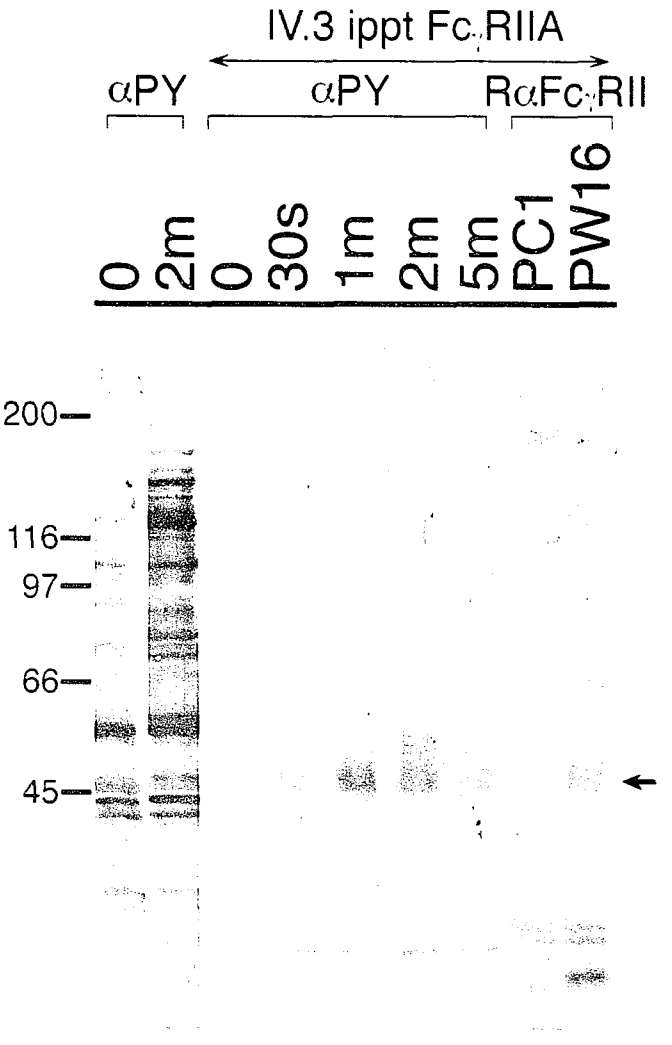


Figure 12

pretreatment apparently abrogated the appearance of the 82,500 M_r band normally induced by FcγRIIA stimulation (Fig. 11, Table 5).

Tyrosine phosphorylation of FcγRIIA. Phosphorylation on tyrosine of FcγRIIA was analyzed by FcγRII-specific immunoprecipitation of cell lysates followed by immunoblotting with mAb 4G10 (Fig. 12). Maximal phosphorylation was seen at 1 min. No basal phosphorylation of the receptor was evident in immunoprecipitates from cells at time 0, nor from mock-stimulated (no IV.3 Fab) or blank-stimulated (IV.3 Fab, but no secondary crosslinking antibody) samples. The identity of the immunoprecipitated 46,000 M_r protein as FcγRIIA was confirmed by co-migration of the tyrosine-phosphorylated bands with an identical complex detected by a polyclonal rabbit anti-FcγRIIA antibody. The 46,000 M_r protein was absent in immunoprecipitates from the FcγRIIA-negative cell line PC1.

Discussion

The T cell and B cell antigen receptors (TCR and BCR), FcγRIIIA, and Fc$_\epsilon$RI are all multi-chain immune recognition receptors (MIRR) (27). One or more subunits of each MIRR share a common tyrosine activation motif in the cytoplasmic domain: D/E-X_7-D/E-X_2-Y-X_2-L-X_7-Y-X_2-L (26) (Fig. 3). The human FcγRIIA and FcγRIIC receptors also share a related cytoplasmic sequence: E-X_8-D-X_2-Y-X_2-L-X_{12}-Y-X_2-L (Fig.3) (86,128). The importance of the MIRR motif is shown by the ability of several chimeric molecules containing the CD3 ζ cytoplasmic domain to transduce signals to T cells following crosslinking (30,266,268). Indeed, crosslinking of a chimera with an 18 amino acid sequence of ζ that contains the acidic tyrosine-leucine motif is sufficient to trigger a rise in

$[Ca^{2+}]_i$ and cytolysis; the two tyrosines were shown by site-directed mutagenesis to be crucial (30,267). A very recent additional study has delineated the functional motif to 17 amino acids (28). Moreover, constructs with triplication of the motif (as occurs in the ζ chain) demonstrated qualitatively identical but quantitatively enhanced signalling, suggesting a redundant role in signal amplification for the three motifs present in ζ. Multiple copies of the motif also stabilized their association with the ZAP-70 tyrosine kinase (of 70,000 M_r) (326,327) which has been implicated in TCR signalling (28). A ZAP-70 homologue, the syk tyrosine kinase, of 72,000 M_r, has been cloned from porcine splenic cells (328). $p72^{syk}$ and the closely related molecule ZAP-70 play central roles in signal transduction via the B and T cell antigen receptors. $p72^{syk}$ is co-precipitated with sIgM (353) and ZAP-70 is thought to be the kinase responsible for phosphorylation of the tyrosines in the ζ chain following TCR activation (28). Immunoprecipitation studies by Zhenghai Shen have identified the 74,000 M_r band, which I had found to be the earliest band induced following huFcγRIIA activation, as syk [Zhenghai Shen, personal communication; (335)]. Syk is a likely candidate involved in $Fc_\epsilon RI$-mediated signalling as well. One of the most prominent proteins phosphorylated on tyrosine upon $Fc_\epsilon RI$ ligation is pp72 (276,280-282). The tyrosine phosphorylation of pp72 is an early event (maximal by 1 min) occurring independently of $[Ca^{2+}]_i$ flux and PKC activity (280) and is not affected by stimulation or inhibition of G proteins (276). The phosphorylation of pp72 was blocked by either RG 50864 (281) and genistein (282), and this inhibition correlated with decreased serotonin and histamine release, whose release from mast cells or basophils via degranulation is one of the end results of $Fc_\epsilon RI$ activation.

Src family kinases are likely to mediate signalling for a variety of MIRRs. Preliminary studies suggest that $Fc_\epsilon RI$ activates the lyn and yes kinases (325,329), and stimulation of FcγRIIIA results in lck activation (315). In B cells, stimulation of the antigen receptor (via either sIgM or sIgD) also results in tyrosine phosphorylation of cellular target proteins, including at least the Ig-α receptor component as well (314,330,331). Again Src family kinases are emerging as potential signal transducing molecules. Blk is solely expressed in B-lineage cells (332), and lyn, which is preferentially expressed in B cells, could be coprecipitated with sIgM of the BCR (333). A more recent study has shown that three Src family kinases, blk, fyn, and lyn, are activated by the BCR and coprecipitate with sIg. In this study, the activation of blk was more marked than of the other two kinases (334).

FcγRIIA, expressed on platelets, monocytes, macrophages, and neutrophils, mediates phagocytosis of immune complexes and is also responsible for the neutrophil superoxide burst (135,209), which is dependent on a flux of $[Ca^{2+}]_i$. Human FcγRIIA, expressed in P388D1 cells, mediates rapid, temperature-sensitive phagocytosis of complexes and $[Ca^{2+}]_i$ flux. Following crosslinking of FcγRIIA expressed in P388D1 cells we observed phosphorylation on tyrosine of post-nuclear cellular proteins that is maximal at 1-2 minutes and then falls to baseline levels by 5-10 min. No additional bands were detectable in whole cell lysates using a 1% SDS lysis buffer (data not shown). The pattern and kinetics of tyrosine phosphorylation we observe in P388D1 cells is very similar to that observed in the human monocytic U937 cell line, activated under similar conditions (Zhenghai Shen,

personal communication). The rapidity of the dephosphorylation that I found is somewhat faster than has been observed in other systems (128), but is in accord with previous work demonstrating the activation of tyrosine kinases following receptor crosslinking. The importance of the kinase activation is indicated by the inhibition of phagocytosis and $[Ca^{2+}]_i$ flux observed after pretreatment of cells with herbimycin A [Table 4, (335)]. Furthermore, nonfunctional FcγRIIA with severe truncations of the cytoplasmic domain expressed in P388D1 cells and wt FcγRIIA expressed in CHO cells do not trigger tyrosine phosphorylation events upon crosslinking, demonstrating that signalling is dependent on both the receptor and cellular components. The total abrogation of any cellular responses to FcγRIIA stimulation following Herbimycin A pretreatment and conversely, the preservation of the tyrosine phosphorylation responses following BAPTA-AM pretreatment together argue that the tyrosine phosphorylation response precedes the $[Ca^{2+}]_i$ flux in response to receptor crosslinking. However, activation of kinases *per se* is not sufficient for functional responses, as the Y252F mutant, which shows an almost complete spectrum of phosphotyrosine--containing proteins upon activation, is functionally inactive. The kinetics of phosphorylation and dephosphorylation may be important as well, as is discussed below.

Oddly, neither splice variant of murine FcγRII activates tyrosine phosphorylation events (125,237,354). This may be due to the insertion of an additional amino acid within the second Y-X-X-L/I sequence in the conserved motif. However, the Δ264 mutant which totally lacks the second Y-X-X-L sequence is able to mediate a partial tyrosine phosphorylation response (Fig. 7). Thus the role of the

second Y-X-X-L sequence appears complex. The b1 and b2 forms of human FcγRIIB, which also do not trigger a $[Ca^{2+}]_i$ flux or cytolysis (216), may be the homologues of murine FcγRII. The ability of murine FcγRIIb2 to localize to coated pits and mediate endocytosis (233,235) may thus reflect other pathways than that utilized by FcγRIIA. The mechanism by which ligation of murine FcγRIIb1 with sIg inhibits activation of B cells (355) and $[Ca^{2+}]_i$ flux (259) is unclear.

It is intriguing that PMA pretreatment increased the phosphotyrosine content of the 85,000 M_r band and abrogated the 82,500 M_r band, both of which show increased intensity on anti-phosphotyrosine Western blots following FcγRIIA crosslinking. However, the increased intensity of the 85,000 M_r band which is presumably mediated through PKC, contrasts with the presence of this band in the Y252F mutant which does not flux Ca^{2+} and therefore presumably does not activate PKC. Thus, the tyrosine phosphorylation of the 85,000 M_r band in response to FcγRIIA signalling probably occurs through a mechanism regulated independently of PKC. In contrast the absence of the 82,500 M_r band in both the PMA pretreated samples and the Y252F mutant is consistent and suggests that PKC may regulate the tyrosine phosphorylation of this 82,500 M_r band. Further work is necessary to define the role of PKC in huFcγRIIA signalling.

Previous work (215) showed that Δ264 was capable of phagocytosis of crosslinked FcγRIIA complexes but failed to mediate a $[Ca^{2+}]_i$ flux, and failed to ingest E-IV.3. Furthermore, the phagocytosis of E-IV.3 by transfectants with wt FcγRIIA was inhibited by BAPTA, showing that for this cell line $[Ca^{2+}]_i$ flux is required for the phagocytosis of large particles. These results have been confirmed

by the correlation of $[Ca^{2+}]_i$ flux with phagocytic index in a series of point mutations of FcγRIIA (335). However, phagocytosis by peritoneal macrophages is not dependent on $[Ca^{2+}]_i$ flux (356,357). These differences may reflect a dependence on membrane recycling in the $P388D_1$ cell line that is not required for peritoneal macrophages because they have a larger area of membrane available for the incoming phagocytic vacuole.

Following crosslinking of the Δ264 FcγRIIA mutant, only a subset of proteins normally phosphorylated on tyrosine following activation of the wt FcγRIIA are observed (Fig. 7; Table 5). Since the Δ264 FcγRIIA mutant phagocytoses immune complexes normally, activation of the phagocytic pathway may be dependent on phosphorylation of some or all of this subset of proteins. The kinetics of the phosphorylation and dephosphorylation by the Δ264 FcγRIIA mutant are similar to wt (Fig. 7, panels A and B, and Fig. 9). The differences in phosphorylation patterns between wt FcγRIIA and the Δ264 mutant suggest that the huFcγRIIA receptor normally signals for the activation of several distinct tyrosine kinases with different specificities. The failure of activation of Δ264 to induce the phosphorylation of $p72^{syk}$ (p74,000 in Fig. 7A; see discussion of $p72^{syk}$, above) argues for this view. Alternatively, one or more of the subset of proteins not phosphorylated in the Δ264 mutant may require direct interaction with epitopes within the carboxyl-terminal 17 amino acids of the wt receptor in order to be phosphorylated.

The residues we chose to mutate were between Arg^{234} to Asp^{264}, a region previously identified as important for phagocytosis of immune complexes (215). Mutation of the acidic clusters at either end of the Arg^{234} to Asp^{264} sequence was

without effect. The importance of the first Y^{252}-X-X-L repeat in the signalling process is shown by the inability of the Y252F FcγRIIA mutant to either phagocytose or flux $[Ca^{2+}]_i$. However mutation of Tyr^{252} to serine or leucine led to FcγRIIA mutants that were fully functional, indistinguishable from wt, even upon analysis of multiple clones of each transfectant. As these results were unexpected, genomic DNA from various Tyr^{252} transfectants was amplified by PCR, subcloned and resequenced by Ching-Tai Lin to confirm the mutations. Although Y252F FcγRIIA was shown to be nonfunctional for $[Ca^{2+}]_i$ flux and phagocytosis (335), crosslinking this receptor led to tyrosine phosphorylation of most of the proteins seen after activation of the wt receptor, with the exception of three major phosphotyrosine--containing proteins of 82,500, 51,000, and 49,500 M_r. p51 has subsequently been identified as $p51^{shc}$ by Zhenghai Shen (personal communication). Shc, which is highly conserved and widely expressed, contains a single SH-2 domain. Shc proteins are thought to be adapters to couple tyrosine kinases to downstream targets that lack SH-2 domains. Shc is associates with and is phosphorylated by the activated PDGF receptor and overexpression of shc results in transformation of fibroblasts (358). Additionally, shc proteins appear to be *in vivo* substrates for v-src and v-fps nonreceptor tyrosine kinases (359). The importance of $p51^{shc}$ in huFcγRIIA signalling is indicated by its absence in both Δ264-- and Y252F--stimulated cell lysates. Neither of these mutant receptors is capable of mediating a $[Ca^{2+}]_i$ flux nor phagocytosis. As the phagocytosis response in these P388D1--derived cell lines is Ca^{2+}--dependent, it is likely that $p51^{shc}$ is a component of the pathway leading to a $[Ca^{2+}]_i$ flux. As was discussed above, $p71^{syk}$ is also a likely

candidate in the $[Ca^{2+}]_i$ pathway. While the rate of phosphorylation induced by Y252F was somewhat slower, the rate of subsequent dephosphorylation was markedly inhibited relative to wt (Fig. 7, panels A and C; Fig. 9). This result suggests that the phosphotyrosine phosphatases responsible for the dephosphorylation are not constitutively active, but are induced as a consequence of crosslinking.

It is not yet clear why the very conservative substitution of Y^{252} with phenylalanine resulted in complete abrogation of $[Ca^{2+}]_i$ flux and phagocytic capacities, whereas serine and leucine substitutions were without effect, but several possibilities can be considered. One speculation is that Phe^{252} results in an *increased* affinity of interaction of the receptor with a target of the signalling pathway, resulting in a "stalling" or "kinetic block" in the pathway, such that the activation of tyrosine kinases continues without the normal kinetics of inactivation through tyrosine phosphatases. When signal transduction components directly interacting with the receptor are identified, it may be possible to demonstrate their differential association with wt versus the Y252F receptor in immunoprecipitaions from ^{35}S-methionine labeled cells. Alternatively, position 252 may be part of an epitope that normally interacts with a phosphotyrosine phosphatase, and the substitution of phenylalanine may prevent this complex formation. Specifically, the aromatic ring of tyrosine or phenylalanine, but not serine or leucine at position 252 may block events required for activation of $p72^{syk}$ and/or of $p51^{shc}$ and, following phosphorylation of Tyr^{252}, the inhibition is removed. Further extensive site--directed mutagenic studies including the substitution of Tyr^{252} with another aromatic amino acid, tryptophan, and additional substitutions of Tyr^{245}, especially with

phenlyalanine, may help substantiate this hypothesis. However, phosphorylation of Tyr^{252} is not needed for activation of tyrosine kinases after crosslinking of the Δ264 mutant, which is not phosphoryated on tyrosine upon activation (335). Analysis of double mutants in which *both* Tyr^{252} and Tyr^{268} are substituted will help delineate the complex relationship between these residues. Additionally, it is important to determine which tyrosine residues are phosphorylated in the wt huFcγRIIA utilizing HPLC or TLC analysis of protease digests of γ-^{32}P-ATP--labeled cells.

Subunit chains of the TCR, BCR, and the $Fc_\epsilon RI$ which contain the MIRR motif are phosphorylated upon receptor aggregation. I found that the huFcγRIIA is also phosphorylated on tyrosine in response to receptor signalling, consistent with one other recent report (128). The receptor phosphorylation kinetics mirrored but did not obviously precede the total cellular tyrosine phosphorylation response. It was intriguing that the phosphorylation of the receptor was not detectable in cellular lysates directly analyzed without immunoprecipitation of the receptor (Fig. 7), in spite of the high level of expression of this receptor by the cell line [1.2×10^6 receptors/cell; see ref. (215)]. One explanation is that the Western analysis of cell lysate was not sensitive enough to allow for the detection of the 15 ng of FcγRIIA receptor calculated to be present in each sample. Alternatively, it may be that only a small portion of the total receptor pool is phosphorylated upon signalling. In that case, either receptor phosphorylation is a byproduct of and is not required for receptor--mediated signalling, or only that small portion of phosphorylated receptors is involved in signal transduction. The percentage of receptor pool phosphorylation can be addressed by serial anti-receptor and anti-phosphotyrosine

immunoprecipitation studies of cells metabolically labeled with ^{35}S-methionine. The requirement of receptor phosphorylation for signal transduction may be addressed through further site--directed mutagenic studies. The work of this laboratory to date implicates Y^{268} as the most likely tyrosine residue to be critically required for receptor signalling, as this residue is absent in the severely impaired Δ264 mutant. However, the limited functions of the Δ264 mutant, which include phagocytosis of complexes and a partial tyrosine phosphorylation response, do not require Y^{268}. The wildtype phenotype of the Y245S mutant suggests that phosphorylation of this residue is not required for the receptor functions we have analyzed. The requirement for phosphorylation of Tyr^{252} may prove to be complex, as was discussed above. Briefly, tyrosine itself may not be requisite at position 252, as serine and leucine substitutions were wildtype. However, the phosphorylation of Tyr^{252} may be necessary to relieve inhibition of receptor signalling interactions imposed by tyrosine's aromatic ring. this was suggested by the severe crippling of receptor signalling resulting form the substitution with $Phe^{252.}$ A more extensive comparison of serine, leucine and phenlyalanine substitutions are all three tyrosine residues of the cytoplasmic domain of huFcγRIIA is clearly warranted.

The results in our P388D1 system contrast somewhat with those of a recent study of huFcγRIIA chimeras studied by vaccinia virus vector mediated transfection of a human cytotoxic T cell line as well as of freshly isolated peripheral human monocytes (216). In this study, both tyrosine residues of the tyrosine activation motif were found to be critical for both $[Ca^{2+}]_i$ flux and ADCC responses. In the P388D1 system, mutation of the first tyrosine of the motif (Tyr^{252}) to serine or

leucine was without effect on any function tested ($[Ca^{2+}]_i$ flux, phagocytosis, and tyrosine phosphorylation) and deletion of the second tyrosine (Δ264) did not totally abrogate receptor function. However, my studies do indicate the importance of the two tyrosine residues. The Y252F mutant was severely compromised, excepting a *nearly* complete tyrosine phosphorylation response, and the Δ264 mutant retained only a limited tyrosine phosphorylation response and the ability to phagocytose complexes but not large particles. While the $[Ca^{2+}]_i$ response of the TCR differs from that of macrophages, exhibiting a prolonged plateau time course essential for other downstream responses, critical cell specific differences between the murine P388D1 macrophage--like cell line and human monocytes are more difficult to appreciate. Further study is indicated, and the vaccinia transfection system will enable the expeditious comparison of huFcγRIIA structure-function relationships in multiple cell lines, which furthermore will enable the assessment of additional responses, such as the superoxide burst.

The results presented in this thesis strengthen the hypothesis that distinct signalling pathways are activated by FcγRIIA for phagocytosis of complexes and for $[Ca^{2+}]_i$ flux. We have shown that protein tyrosine phosphorylation is a prerequisite for both of these functions. Furthermore, the 74,000 Da protein, subsequently identified by Zhenghai Shen as $p72^{syk}$, which is one of the earliest proteins phosphorylated on tyrosine following activation of wt FcγRIIA, is not phosphorylated after activation of Δ264 and only minimally by Y252F. Since Δ264 phagocytoses complexes normally, this suggests that $p72^{syk}$ plays no role in phagocytosis, but is important for $[Ca^{2+}]_i$ flux. The 51,000 Da protein which is

absent from the responses of both Δ264 and Y252F, has been identified by Zhenghai Shen as p51^{shc}, and may serve to couple upstream an upstream tyrosine kinase with downstream effectors of the $[Ca^{2+}]_i$ flux. Lastly, the delayed kinetics of the dephosphorylation in the face of a nearly complete phosphorylation response in the severely crippled Y252F mutant suggest that PTPases play a central role in huFcγRIIA signal transduction. Additional studies are needed to identify the kinases and phosphatases involved in these pathways.

Materials and Methods

Reagents. MAb produced by the IV.3 hybridoma (which is obtainable from ATCC) was purified from spent medium by chromatography on a protein G-Sepharose column. The Fab was prepared by digestion with immobilized papain (Sigma, St. Louis, MO) and purified using a Protein A (Pierce, Rockfield IL) column and mono Q chromatography (Pharmacia, Piscataway, NJ). The anti-phosphotyrosine mAb 4G10 was purchased from UBI (Lake Placid, NY), and was also provided as a generous gift of Dr. Thomas Roberts (Dana Farber Research Institute, Boston, MA). Alkaline phosphatase--conjugated secondary antibodies were from Organon-Technica Cappel (Durham, NC). Unconjugated and biotinylated goat anti-mouse IgG $F(ab')_2$ were purchased from Jackson Immunoresearch (West Grove, PA). Herbimycin A (260) was very kindly provided by Dr. Yoshimasa Uehara. Bapta-AM was purchased from Molecular Probes, Inc. (Eugene, OR).

Cell lines and culture conditions. The P388D1 transfectants expressing huFcγRIIA and the truncation mutants Δ207, Δ233, and Δ264 were established and initially analyzed by Dr. Joseph A. Odin, as was the huFcγRIIA-expressing CHO cell line (215). The location of the truncations is illustrated in figure 6 and the functional analysis of these mutants and wt receptor is presented in Table 4. Point mutants, illustrated in Figure 6, were constructed by Ching Tai Lin, who also performed analysis of their capacities for phagocytosis of complexes and erythrocytes as well as $[Ca^{2+}]_i$ flux, and these results (335) are summarized in table 4. All P388D1-derived cell lines were cultured in Dulbecco's Modified Eagle's Medium

(DMEM) (Gibco Laboratories, Grand Island, NY) supplemented with 5% FCS (Intergen, Purchase NY or Flow, McLean, VA) and 200 μg G418 (Geneticin) (Sigma) and maintained at 37°C with 20% O_2, 7% CO_2 in a humidified Queue incubator (Parkersburg, WV). Cells were passaged and collected for experiments by gentle repeated pipetting when plates were near confluency. The CHO cell line was maintained in hypoxanthine--deficient DMEM supplemented with 10% dialyzed FCS, 16 μM thymidine and 300 μM proline and were passaged following 1X trypsin digestion (Sigma).

Western Blot Analysis of Tyrosine Phosphorylation. Cells were collected from plates by gentle pipetting with DMEM, 2% FCS, 20 mM Hepes, pH 7.0 and dispensed at 2 x 10^6 cells/eppendorf tube. Gently pelleted cells were resuspended in 100 μl DMEM containing 20 mM Hepes, pH 7.0, and mAb IV.3 Fab (2 μg/ml, 30 min, 37°C), washed, and stimulated in the same medium containing 100 μl of goat anti-mouse IgG $F(ab')_2$ (40 μg/ml, 37°C). In mock stimulations mAb IV.3 Fab was omitted. In "blank" stimulations the secondary crosslinking goat anti-mouse IgG $F(ab')_2$ was omitted. Mock stimulations were performed in all experiments for each time point. The stimulation was ended by pipetting the cell suspension (~100 μl) into 1.2 ml of PBS-2% FCS prechilled in a salt-ice-water bath. Following centrifugation for 10 sec, pellets were lysed (100 μl, 0.5% NP-40, 0.1% sodium deoxycholate, 10.0 % glycerol, 20 mM Na-PO_4, pH 7.8, 70 mM NaCl, 50 mM NaF, 400 μM Na_3VO_4, 5 mM EDTA, 1 mM PMSF , and 10 μg/ml each of aprotinin, leupeptin, soybean trypsin inhibitor, and pepstatin A) and cleared by centrifugation (20,000 x g, 20 min, 4°C). To each 15 μl of lysate (3 x 10^5 cell

equivalents), 5 μl of 4X SDS sample buffer (8% SDS, 30% glycerol, 0.03% bromophenol blue) was added and samples were immediately boiled for 1 min prior to gel loading. Following electrophoresis (generally at 60-110 Volts, constant voltage mode, for 12-20 hours) of cleared lysates on a 7-17% SDS-polyacrylamide gel (360), gels were electroblotted in Tris-glycine buffer (at 60 Volts for 6.5 hrs) onto nitrocellulose following the "Western blot" method of Towbin (361). Blots were blocked overnight at 4°C in TBS, 5% BSA (Sigma), 0.05% Tween-20, and 0.02% Azide. All subsequent steps were performed at rt. The blots were incubated with 1 μg/ml of the 4G10 anti-phosphotyrosine mAb (1 μg/ml, TBS, 1% BSA, 2 hr). Following three washes, the 4G10 mAb was detected by incubation with alkaline phosphatase--conjugated goat anti-mouse IgG $F(ab')_2$ at 1:1000 dilution in TBS 1% BSA, washed, and developed as described previously (153). The nitrocellulose blots were digitized, and densities of individual bands were quantified using NIH Image 1.43 software.

Pharmacologic Studies. Cells were preincubated with herbimycin A (260) (10 μM, 16-20 hr, 37°C), a kind gift of Dr. Yoshimasa Uehara (National Institute of Health, Tokyo, Japan). Cells were pretreated with PMA at 100 μM during the staining with mAb IV.3 for either the entire 30 minutes or the last 5 minutes of the staining incubation, which resulted in either 30 min or 5 min pretreatment with PMA prior to stimulation by FcγRIIA crosslinking. BAPTA-AM (353) at 100 μM was preincubated with cells during the mAb IV.3 staining for 30 min at 37°C. In all pharmacologic studies, the agent used for pretreatment was continuously present during stimulation.

Immunoprecipitation of FcγRIIA. Mock and stimulated cell lysates from 5 x 10^6 cells were obtained as detailed above, using a biotinylated secondary crosslinking antibody. This enabled subsequent immunoprecipitation with streptavidin-conjugated agarose. [Prior to immunoprecipitation, a small aliquot of lysate (4 x 10^5 cell equivalents) was removed for analysis as above to demonstrate stimulation of the cellular lysate.) Following overnight incubation with the streptavidin agarose, immunoprecipitates were washed 5 times in cold lysis buffer and released by boiling in SDS sample buffer. Samples were equally divided onto two electrophoresis gels (2.1 x 10^6 cell equivalents per lane) and one blot was probed for phosphotyrosine. The second blot was probed for FcγRIIA with a polyclonal rabbit anti-FcγRIIA elicited against a recombinant truncated human FcγRIIA protein containing only the extracytoplasmic domain (362). The rabbit anti-FcγRIIA IgG was used at a concentration of 10 μg/ml in TBS, 1% BSA, 0.5% NP-40, and detected using an alkaline phosphatase--conjugated goat anti-rabbit IgG antibody $F(ab')_2$

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