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Signal transduction and structure function studies of human FcγRIIA

Lin, Ching-Tai, Ph.D.

City University of New York, 1994

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Signal Transduction and Structure Function Studies of Human FcgammaRIIA

by

Ching-Tai Lin

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.

1994

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Abstract

Signal Transduction and Structure Function Studies of Human FcgammaRIIA

by

Ching-Tai Lin

Advisor: Jay Unkeless, Ph.D.

Receptors for the Fc domain of IgG (FcγR) on leukocytes mediate a pleiotropic response following crosslinking by immune complexes. Signaling events mediated by crosslinking of human FcγRIIA and FcγRIIA mutants were analyzed in transfected $P388D_1$ mouse macrophage cell lines. Activation of wild type FcγRIIA in transfected $P388D_1$ cells led to rapid and transitory tyrosine-phosphorylation of several proteins, including the tyrosine kinase $p72^{syk}$. We analyzed mutants in both Y-X-X-L motifs of the FcγRIIA cytoplasmic domain. Deletion of the COOH-terminal motif, mutation of Tyr^{268} to Phe, Leu, or Ser, and mutation of Leu^{271} to Ala resulted in FcγR's that did not activate $p72^{syk}$, flux $[Ca^{2+}]_i$, or phagocytose IgG sensitized erythrocytes, but which were competent to internalize immune complexes and to phosphorylate on tyrosine a subset of the normal substrates. Mutation of the NH_3-terminal motif was more complicated. Mutation of Tyr^{252} to Ser or Leu did not alter the phenotype. However, mutation of Tyr^{252} to Phe, or Leu^{255} to Glu resulted in

a severely impaired phenotype, unable to flux $[Ca^{2+}]_i$, lacking most (but not all) tyrosine kinase activity, and capable of internalizing complexes only after extensive crosslinking. In contrast, deletion of both motifs resulted in a completely inactive receptor, unable to activate tyrosine kinases, to flux $[Ca^{2+}]_i$, or to internalize complexes.

Crosslinking FcγRIIA led to the transient generation of inositol trisphosphate, $[Ca^{2+}]_i$ flux, and rapid tyrosine phosphorylation of cellular substrates, including Shc, PLC-γ1, and a tyrosine kinase $p72^{syk}$. In contrast, no tyrosine phosphorylation of Shc or PLC-γ1 was detected in cells transfected with mutant receptors that failed to trigger $[Ca^{2+}]_i$ flux. PMA inhibits both tyrosine phosphorylation of Shc and IP3 production leading to $[Ca^{2+}]_i$ flux. However, PMA does not affect tyrosine phosphorylation of PLC-γ1 and $p72^{syk}$. These results suggest that tyrosine phosphorylation of Shc and PLC-γ1 is important for the initiation of $[Ca^{2+}]_i$ flux, and that activation of protein kinase C (PKC) may modulate the activity of PLC-γ1.

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I. Background and Significance

Overview of receptors for IgG

Fc receptors for IgG (FcγRs) bind IgG, IgE, and IgA via the Fc domain, and form a subfamily within the immunoglobulin gene superfamily. 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 immune 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 their state of activation, inducing the transcription and synthesis of lymphokines such as TNF-α and GM-CSF, and receptors for 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) (1), and coaggregation of the B cell antigen receptor with FcγRs inhibit B cell differentiation (2). The FcγR on placental syncytiotrophoblasts (3) may be involved in transcytosis of immunoglobulin. The presence of high titers of anti-FcγR Ig (4,5) have been reported in both human and murine autoimmune disease. Probably the ligation of FcR by immune complexes plays a major role in inflammation in rheumatoid arthritis and systemic lupus erythematosus by activating production of inflammatory cytokines.

$Fc\gamma Rs$ differ from growth factor receptors in that crosslinking or immobilization of the membrane receptors by polyvalent immune complexes, rather than binding of ligand *per se* is the triggering event. 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 [see (6-8) for reviews]. There may, however, exist preferred orientations of receptors engaged in signaling since some mAbs directed against $Fc_{\epsilon}RI$ are not capable of initiating signaling when used to crosslink the receptor (6).

All FcRs except CD23 ($Fc_{\epsilon}RII$) are members of the Ig supergene family(9). With the exception of $huFc\gamma RIIIB$, which is anchored in the neutrophil plasma membrane by a glycan phosphatidyl inositol (GPI) moiety (10), all $Fc\gamma Rs$ are class I membrane glycoproteins. Low avidity forms of membrane bound $Fc\gamma Rs$ contain two extracellular Ig-like regions, whereas high avidity forms contain three Ig-like regions. Assigning functions to individual $Fc\gamma Rs$ has been a challenging task since within a subclass receptors share immunologically indistinguishable extracellular domains and their cellular distributions overlap considerably. Elucidation of the receptor-mediated signaling pathways has been an exciting area with the recent realization that varied immune recognition receptors share a common motif (11,12). This motif has been shown in the T cell receptor (TCR) system to be necessary and sufficient for signal transduction (13).

Heterogeneity and genetic polymorphisms of $Fc\gamma Rs$

$HuFc\gamma RI$ (CD64)

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. The purified receptor has a M_r, determined by SDS-PAGE, of 72,000 (7) (Table 1), which decreases after removal of N-linked carbohydrate to 40,000-50,000. Very recent work has identified three highly homologous genes FcγRI genes (A, B, and C) which encode four huFcγRI transcripts, a1, b1, b2, and c1 (14). The FcγRIA gene product, which was originally cloned using a eukaryotic expression shuttle vector (15), 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. In contrast, huFcγRII and huFcγRIII encode only two Ig-like domains. The FcγRIB gene gives rise to two transcripts, one encoding a transmembrane protein identical to that of the FcγRIa1 transcript, but lacking the third membrane-proximal extracellular domain that is believed to endow high affinity binding of IgG (16). Another transcript of the FcγRIB gene, as well as the FcγRIc1 transcript encode secreted forms of the receptor that lack any transmembrane and cytoplasmic domains (14). The transmembrane domain of the FcγRIa1 product, which appears to be the receptor previously identified with various antibodies (14), is 21 residues long and the cytoplasmic domain is short and highly charged (15,17). 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 (15). FcγR probably have redundant functions since several members of a Belgian family have a complete absence of huFcγRI expression on their peripheral blood monocytes (18), and are

apparently healthy.

HuFcγRII (CD32)

A second subclass of human FcγR, huFcγRII (CD32), was initially identified by affinity chromatography of U937 lysates on IgG-Sepharose. The anti-huFcγRII mAb IV.3 immunoprecipitates an antigen of about 40 kDa. HuFcγRII is found on monocytes, neutrophils, platelets, B cells, eosinophils, basophils and trophoblasts. 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. The affinity with which huFcγRII binds human IgG subclasses is $IgG_1 = IgG_3 >> IgG_2 = IgG_4$ (7) (Table 2).

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 (19), 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 (20). Initial cDNA clones of huFcγRII appeared to be nearly identical products of a single gene with no differential splicing (21). Subsequently, additional cDNA clones were isolated showing that at least three genes encode huFcγRII proteins (22). The nomenclature is summarized in Table 1.

FcγRIIB sequences differ from FcγRIIA in the signal sequence and in part of the cytoplasmic domain while FcγRIIB differs from FcγRIIC 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 by their signal sequences: that of FcγRIIA is homologous to the murine FcγRIII signal sequence, while the signal sequence of FcγRIIC is homologous to that of murine FcγRII. Otherwise, FcγRIIA and FcγRIIC are nearly identical. The cytoplasmic domain of FcγRIIB, 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 (22,23).

Monoclonal Abs are not available that discriminate rigorously between the three major isoforms of huFcγRII, owing to the great homology of their extracellular domains. In addition to the isotypic variation, there are also FcγRIIA allotypes HR (high responder) and LR (low responder) that differ in their ability to bind murine IgG1. The HR allotype that binds murine IgG1 has Arg_{133} substituted for His_{133} (24,25), resulting in a slightly different isoelectric focusing pattern (26). However, $Fc\gamma RIIA^{LR}$ allotype interacts efficiently with human IgG2 whereas the $Fc\gamma RIIA^{HR}$ form binds IgG2 very poorly, suggesting that FcγRIIA may somehow regulate hIgG subclass production/turnover (27).

HuFcγRIII (CD16)

HuFcγRIII (CD16), binds IgG1 and IgG3 with an intermediate affinity (K_a of ~4 x 10^6 M^{-1}) and is expressed on macrophages, NK cells, neutrophils, eosinophils, and some T cells. HuFcγRIII on SDS-PAGE runs as a broad smear of 50,000-70,000 M_r, probably due to glycosylation heterogeneity. 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 (7). Subsequent cDNA cloning experiments demonstrated that the NK cell transcribes an mRNA distinct from that of neutrophils (28,29). There are two FcγRIII genes, huFcγRIIIA, expressed in NK cells and macrophages and huFcγRIIIB, expressed in neutrophils.

Initial attempts to express huFcγRIIIA in fibroblast lines were unsuccessful. FcγRIIIA has an unusual eight amino acid stretch which includes a charged aspartyl residue in the 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 (6,30). NK cells which only express FcγRIIIA, contain γ and ζ transcripts. Cotransfection of FcγRIIIA and γ cDNAs resulted in 50-100 fold increase in surface expression of FcγRIIIA in COS-7 cells and physical association of FcγRIII with γ was demonstrated in a murine macrophage line (31). In NK cells, huFcγRIIIA associates predominantly with γ_2, but also with ζ_2, ζ-γ, and γ-η dimers (32). These three subunits, γ, η, and ζ, are members of the newly defined zeta gene family.

HuFcγRIIIB is anchored to the neutrophil cell membrane via a GPI linkage and can be released from the cell membrane by phosphoinositol-specific phospholipase C (33). 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 (34), 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 FcγRIIIB (33). There is, however, some evidence that not all GPI-anchored proteins in PNH are reduced parallel is not equal (35), since FcγRIIIB expression is maintained at low levels in the total absence of the GPI-anchored protein DAF (CD55) and CD59 (35).

Two allotypes (NA1 and NA2) exist for huFcγRIIIB. These allotypic differences can cause autoimmune neutropenia in infants (36). Two receptor forms (M_r 19,000 and 21,000) on neutrophils were distinguished after deglycosylation followed by SDS-PAGE (37). 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. FcγRIIIA, which is not polymorphic, always types as NA2.

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 (38). 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 (39-42). A study utilizing

monomeric IgG2a to determine the kinetic parameters of the muFcγRI found that the Ka varied from $2.2x10^{-7}$ at 37°C to $1.1x10^{-8}$ at 4°C, indicating that the interaction is exothermic (43). 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°C of 2.6 min. Thus, muFcγRI is in rapid equilibrium with IgG2a in the local environment (43). The number of high affinity IgG2a binding sites measured at 4°C is 84,000 on $P388D_1$ cells, 110,000 on normal peritoneal macrophages, and 440,000 on thioglycollate--stimulated macrophages. The muFcγRI is trypsin-sensitive (41,43).

MuFcγRI has been cloned (17), and is closely related to huFcγRI (15). 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, has a Ka of $\sim 5x10^7$ M^{-1} at 0°C for IgG2a (17), which is consistent with that obtained the receptor on macrophages (43). Southern analysis suggests that the receptor is encoded by only one gene (17).

MuFcγRII/MuFcγRIII

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 (41,44-46), and ligand binding (39,40,42) as well as the isolation of FcγRII/III loss

variants (41) 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 (47) and binds with a Ka of 9.6×10^8 M^{-1} (48), and is now known to recognize both muFcγRII and muFcγRIII. 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 (49). Various expression studies of the cloned cDNAs for muFcγRII have confirmed that the ligand specificity of the recombinant truncated receptor (50), as well as of that expressed on the cell surface (51,52), includes all murine isotypes except IgG3. The relative ligand binding affinity of a recombinant truncated (secreted) form of muFcγRII is IgG1 > IgG2b = IgG2a (50). The mAb 2.4G2 purification results agree closely with other work in which FcγR with ligand binding activity was isolated (53,54).

The cDNAs encoding muFcγRII and muFcγRIII were cloned by three groups (23,51,55). 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 (50,56) and each Ig-like domain has two sites of N-linked glycosylation (56). 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 (23). 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 (23). Analysis of overlapping λ genomic clones of muFcγRII revealed at least 10 exons covering $\sim$15 kb (57). A novel cytoplasmic exon was also found (57), 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 (23). 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 (23). MuFcγRIIb1 is expressed in both myeloid and lymphoid cells, while expression of muFcγRIIb2 is limited to macrophages and macrophage cell lines (23,52,58). A muFcγRII transcript of undetermined splice form is detected in a mastocytoma line (58).

There is a highly conserved sequence in the transmembrane domains of muFcγRIII, huFcγRIIIA, and the α subunit of the high affinity rat FcγR_{ϵ}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 (23,28). Expression of the muFcγRIII gene, similar to the huFcγRIIIA gene, is dependent on coexpression of the γ chain (31), and this receptor is the only FcγR expressed in NK cells where FcγRIIIA mediates antibody-dependent cell-mediated cytotoxicity (ADCC) (59).

The muFcγRIII gene is expressed in macrophages and macrophage lines, a mastocytoma line, NK cells, and mesangial cells (52,59-62). Two reports confirm that the muFcγRIII gene is in fact translated and expressed on the cell surface (60,61); both groups utilized an FcγRIII-specific anti-peptide antisera. The muFcγRIII protein has a M_r on SDS-PAGE of 45-55 kDa (63). The effect of a panel of lymphokines on muFcγRIII expression was studied (60) using the J774a cell line which does not express either muFcγRII message (52). Only IFNγ and interleukin 6 (IL6) resulted in increased binding of mAb 2.4G2 (60). 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 (60). The presence of other muFcγR genes is suggested by the isolation of a cDNA clone which shares amino acid homology with muFcγRIII and muFcγRII (57).

Table 1. Properties of FcγR

Receptor	Mol. Wt. (kDa)	Cell Distrib.	Affinity	mAbs
huFcγRIA,B,C	72	monocyte, macrophage, U937, HL60, INFγ treated neutrophils	high	32.2, 62, 22, 44, 10.1, FR51
huFcγRIIA	40	U937, monocyte, neutrophil, platelet	low	IV.3, 2E1, KB56 41H.16
huFcγRIIB$_1$-B$_3$	40	B cell, U937, monocyte, neutrophil placenta	low	KB61, 41H.16, 2E1, IV.3
huFcγRIIC	40	B cell, U937, monocyte, neutrophil	low	KB61, 41H.16, 2E1, IV.3
huFcγRIIIA	50-70	monocyte, NK, macrophage	medium	3G8, CLB-FcR-GRAN1, B73.1 Leu-11a, Leu-11b, Leu-11c
huFcγRIIIB	50-70	neutrophil	low	same as above
muFcγRI	?	macrophage	high	(none)
muFcγRIIb$_2$	50-70	macrophage, a mastocytoma line NK, mesangial cells	low	2.4G2
muFcγRIIb$_1$	50-70	lymphoid and myeloid cells	low	2.4G2
muFcγRIII	50-70	macrophage, NK	low	2.4G2

Table 2. Ligand specificity of FcγRs

Receptor	Ligand specificity (IgG isotypes) Mouse	Human
huFcγRI (CD64)	2a=3>>1, 2b	1=3>4>>2
huFcγRII^{LR} (CD32)	2b>>2a, 3>1	1, 2, 3>> 4
huFcγRII^{HR}	1, 2b>>2a, 3	1=3>>2, 4
huFcγRIII (CD16)	3>2a>2b>>1	1, 3>>2, 4
muFcγRI	2a, 2b>>1, 3	3>1>4>2
muFcγRIIb$_1$	2b>2a>1>>3	3>1>4>>2
muFcγRIIb$_2$	2b>2a>1>>3	3>1>4>>2
muFcγRIII	2b>2a>1>>3	3>1>4>>2

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 (64-66). In addition, IFN-γ (50 ng/ml) treatment of neutrophils, which normally do not express huFcγRI, resulted in the expression of ~13,600 FcγRI molecules per cell (65). *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 (67). Streptococcal pharyngitis can result in modest elevations of FcγRI number on monocytes and more dramatic levels on neutrophils (68). 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 (69). However, IFN-γ decreased the expression of $Fc_\alpha R$ in these cells.

Glucocorticoid therapy decreases FcγRI expression on monocytes *in vivo* (70) but is reported to have no effect *in vitro* (71). 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 (72). Dexamethasone augments the IFN-γ stimulation of FcγRI expression on monocytes (71,73), but abrogates the effect of IFN-γ on HL-60 cells and neutrophils (73,74).

Monocytes usually have little FcγRIIIA (75), although there is a small percentage of monocytes that express FcγRIIIA (76). 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 (77). 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 (77).

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 (60). 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 (78). However, elevation of cAMP in neutrophils by treatment with forskolin markedly inhibited phagocytosis of IgG opsonized particles (79). 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 (80). 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 (81).

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 (77), 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 (82,83). TNF-α (84,85) similarly will prime neutrophils for enhanced superoxide production following stimulation by fMLP. These results are in agreement with others (86) showing that GM-CSF enhances cytotoxic potential of ADCC by neutrophils and eosinophils without altering FcγRII expression.

Release of cytokines following activation 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, and lymphotoxin (87,88). 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 (89,90). This provides a degree of control for the elaboration of potentially deleterious cytokines,

restricting optimum secretion to the locus of inflammation and hydrolase release.

Association of FcγRI and FcγRIIIA with γ and ζ chains

The mechanisms of FcγR signaling following binding of immune complexes are the subject of intensive study. However, recent work has shown that in addition to FcγRIIIA, FcγRI is also associated with a γ subunit (91). The 7,000 M_r γ subunit exists as a disulfide-linked homodimer, and is present in FcγRIIIA and $Fc_{\epsilon}RI$ receptor complexes. 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. This sequence has been implicated in mediating the association of these receptors with the γ subunit (92,93). 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 (94). A sequence present in the transmembrane domain of huFcγRI shares identity with the γ transmembrane sequence at five of the ten residues and has conservative replacements in the others: GIMFLVNTVL. The most notable difference is the substitution of an asparagine for a negatively charged aspartate residue. Mutation of the aspartate to valine in the TM of huFcγRIIIAα lessened the dependence on γ chain for receptor expression (95). This may explain why huFcγRI is not dependent on coexpression of the γ chain for surface expression (14).

Various homodimers and heterodimers of the zeta family are associated with the TCR, BCR, FcεRI, and FcγRIIIA receptors and are involved in the signal transduction pathways of these receptors (96). Each of these receptors 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 (12) and contain TAM (tyrosine-based activation motif) or ARH1 (antigen receptor homology 1) motifs (97,98) (Fig. 1A). 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. A variant of the MIRR motif differing 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 (99). The expression of a polypeptide containing a single copy of the motif is sufficient for many aspects of MIRR signaling. The evolution of multisubunit signaling complexes with duplication of signaling units may be particularly suited for antigen receptors whose ligands are diverse, resulting in similarly diverse patterns of receptor orientation upon antigen-mediated receptor crosslinking (12). The structure-function relationships and evolutionary implications of this remarkable conservation of both motif and subunit structure among complex and functionally related receptors are elegantly addressed by Keegan and Paul (12).

Fc receptor crosslinking activates tyrosine kinases

The earliest event in MIRR signaling is the activation of at least one nonreceptor tyrosine kinase. The kinetics of tyrosine phosphorylation precede phosphatidylinositol (PI) hydrolysis (100) and tyrosine kinase inhibitors such as genistein and herbimycin A completely block the ability of the TCR to stimulate PI turnover (101). The kinetics of tyrosine phosphorylation stimulated via the BCR, $Fc_{\epsilon}RI$, and FcγRs is also rapid and transient and is independent of $[Ca^{2+}]_i$ flux or

PKC activation (102). Tyrosine kinase inhibitors inhibit $Fc_{\epsilon}RI$-mediated degranulation (103) and FcγRIIIA-mediated $[Ca^{2+}]_i$ flux, PI turnover (102), ADCC, and NK killing (104).

The TCR signaling pathway is the most extensively studied to date. The src family member $p59^{fyn}$ co-precipitates with the TCR (105). Furthermore, T cells from mice in which $p59^{fyn}$ was knocked out did not proliferate following treatment with a combination of phorbol ester plus either anti-CD3 antibody or concanavalin A (106). The src family kinase $p56^{lck}$ is tightly associated with CD4 and CD8 (107), and a T cell line defective in Lck expression was unresponsive to TCR crosslinking (108). However, TCR ligation does not activate $p56^{lck}$ (109) and the TCR- and CD4- induced protein tyrosine phosphorylation patterns are different (110). Thus while Lck may not be the proximal transducer of the TCR signal, it nevertheless plays an important regulatory role. 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 (111).

Src family kinases are likely to mediate signaling for a variety of MIRRs. In B cells, stimulation of the antigen receptor (via either sIgM or sIgD) also results in tyrosine phosphorylation of cellular target proteins, including Ig-α (112). Blk, solely expressed in B-lineage cells (113), and Lyn, preferentially expressed in B cells, were coprecipitated with sIgM (114). A more recent study has shown that three Src family kinases, Blk, Fyn, and Lyn, are activated by the BCR and coprecipitate with sIg (115).

Src-like kinases are also associated with Fc receptors. Recent study has shown that $p56^{lck}$ is activated and co-immunoprecipitated with FcγRIIIA in NK cells following crosslinking by anti-FcγRIIIA antibody or immune complexes (116). Fgr, a Src-like PTK expressed in granulocytes, monocytes, and natural killer cells, is associated with FcγRII and involved in signal transduction in neutrophils (117). $Fc_{\epsilon}RI$ activates the Lyn and Yes kinases (118). Stimulation of FcγRIIIA results in Lck activation (102). A PTK homologous to ZAP-70, $p72^{syk}$ has been cloned from porcine splenic cells and shown to be co-immunoprecipitated with membrane IgM and activated following the engagement of membrane IgM (119). Activation of the high affinity FcϵRI results in phosphorylation and activation of $p72^{syk}$ (120). Recently, $p72^{syk}$ has been identified as a tyrosine kinase activated by FcγRI and FcγRII (121).

Protein tyrosine phosphatase and receptor-mediated signaling

Following MIRR-stimulated phosphorylation, dephosphorylation occurs, suggesting involvement of phosphatases in these signaling 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 signaling, and CD45 may modulate the cellular response to $Fc_{\epsilon}RI$ and BCR. T cells lacking CD45 are unable to proliferate in response to CD3 crosslinking, but a CD45-expressing revertant was able to respond (122). CD45 has been shown to be associated with the TCR (123) and modulates the tyrosine kinase activity of $p56^{lck}$ (124). In a $CD45^-$ plasmacytoma cell line, the ability to mobilize Ca^{2+} when sIgM of the BCR was ligated was restored by transfection of CD45. Moreover, CD45 was

shown to regulate signal transduction by modulating the phosphorylation state of the Ig-α and Ig-β subunits both *in vitro* and *in vivo* (125). Treatment of human basophils with anti-CD45 mAb inhibited the $Fc_{\epsilon}RI$-mediated release of histamine (126). The exact nature of the role of CD45, either as a regulatory or direct component of the various MIRR signaling 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. The roles and identities of protein tyrosine phosphatases in FcγR signaling remain to be elucidated.

Activation of downstream signaling proteins

Crosslinking of Fc receptor results in tyrosine phosphorylation of cellular substrates. Studies of signaling pathways of growth factor receptors have shown that the downstream effector proteins usually contain SH2 and/or SH3 domains. The SH2 (Src homology region 2) domain is a noncatalytic region of approximately 100 amino acids. A wide variety of proteins involved in intracellular signal transduction, notably the non-receptor protein kinases, contain this conserved domain (127), which plays an important role in the intracellular responses to growth factor stimulation by binding to phosphotyrosine containing proteins (128). Different SH2 regions have specificity for binding to particular tyrosine phosphorylated sites, as has been demonstrated for the binding of p85 subunit of PI3'-kinase, GTPase activating protein (GAP) and PLC-γ to activated growth factor receptor (129-131). Mutations in the SH2 region have been shown to cause dramatic changes in biochemical properties and biological functions of SH2-containing proteins such as Src, Abl and Crk (132-134).

The SH3 domain, another conserved sequence of approximately 60 amino acids, is often (but not exclusively) found in proximity to SH2 domains. The SH3 domains bind to a proline-rich motif (135), and are involved in the control of small Ras-like guanine nucleotide-binding (G) proteins. The SH3 domain of GAP has been identified as an essential sequence for ras-GAP-mediated signaling (136). Studies in vulval development in the nematode, *Caenorhabditis elegans* showed that an SH3-containing protein, Sem5, is crucial to that process and that the SH3 domains are critical for the function of Sem5 (137).

$Fc_{\epsilon}RI$ 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 (138). The receptor phosphorylation is rapidly reversible upon receptor disengagement (139). $Fc_{\epsilon}RI$ crosslinking results in tyrosine phosphorylation of PI-PLC-γ1 (138). Activation of PLC-γ1 is known to occur through phosphorylation of multiple tyrosine residues without the participation of G proteins (140). Serine phosphorylation of PI-PLC-γ1 by PKA (cAMP-dependent kinase) and PKC may serve to modulate the interaction of the enzyme with tyrosine kinases or phosphatases (141). 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 (142).

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 cleaves primarily phosphatidylcholine (PC) to yield phosphatidic acid (PA), which 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 $Fc_{\epsilon}RI$ (143). 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 (144). 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 (145). The role of PLD in FcγR signaling remains to be determined.

The downstream targets following FcγR crosslinking are less well known compared to the growth factor receptor system. Activation of FcγRIIIA expressed in the natural killer (NK) cells led to the tyrosine phosphorylation of PLC-γ1, PLC-γ2 and $p56^{lck}$ (102,146). HuFcγRI and huFcγRII upon activation mediate the tyrosine phosphorylation of PI-PLC-γ1. Pretreatment with herbimycin A abolished this phosphorylation as well as phosphatidylinositol (PI) turnover (147).

Signaling by FcγR isoforms and mutants

HuFcγRIIA has been demonstrated to mediate phagocytosis when transfected into COS-1, 3T6 murine fibroblasts, and the P388D_1 murine macrophage-like cell line (148,149), but was unable to do so in CHO fibroblasts (149). HuFcγRIIB (b1 form), which does not contain the MIRR motif, was unable to mediate phagocytosis in COS cells (150), nor does it trigger $[Ca^{2+}]_i$ flux (151). While both FcγRII and FcγRIII are expressed on murine mast cells, the degranulation response induced in these cells by crosslinking with the mAb 2.4G2 may be solely mediated through muFcγRIII. Only muFcγRIII, but not muFcγRIIb1 or muFcγRIIb2, is able to mediate serotonin and TNF-α release when transfected into a rat basophilic leukemia cell line (RBL-2H3) (152). Identical results were obtained when $[Ca^{2+}]_i$ flux, phosphoinositide hydrolysis, release of arachidonate metabolites, and protein tyrosine phosphorylation were assessed (104).

However, 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 (153). Both forms of the receptor will direct *Toxoplasma gondii* opsonized with IgG to lysosomes (154). 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. 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 FcγRIIb1 and FcγRIIb2 forms of muFcγRII were capable, when co-aggregated 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 (155). 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 (156).

A deletion mutant of huFcγRIIA (Δ264) lacking the 17 carboxyl-terminal residues (including the ultimate Y-X-X-L of the motif) was still able to mediate phagocytosis of receptor-bound immune complexes, but failed to mediate $[Ca^{2+}]_i$ flux associated with activation of the wt FcγRIIA (149). However, the Δ264 mutant did not trigger the phagocytosis of 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. 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 (151). 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 (157), which differs from the previous two studies just discussed (149,151).

GPI anchored FcγRIIIB

FcγRIIIB, a molecule with a glycan phosphatidyl inositol (GPI) anchor at the carboxyl terminus expressed exclusively on neutrophils (28,158), 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 (159). FcγRIIIB ligation has been demonstrated to trigger the release of hydrolases, but apparently cannot stimulate a respiratory burst (160). 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 (161). Similarly, cleavage of FcγRIIIB by elastase, leaving FcγRII intact, does not alter the superoxide burst (162). The

blockade of FcγRII on neutrophils by mAbs inhibits the respiratory burst, which cannot be triggered by crosslinking of neutrophil FcγRIIIB alone (163).

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 (164,165) suggest that huFcγRIIIB 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 (166). 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 (166). A patient with SLE was found who did not express huFcγRIIIB on her neutrophils, due to a probable deletion of the huFcγRIIIB gene (167). The patient's neutrophils did have reduced ability to rosette IgG-coated E, as suggested by earlier studies of neutrophil function (164,165). However, this patient did not exhibit any unusual susceptibility to bacterial infections, and the levels of other GPI-linked proteins and huFcγRII were normal.

FcγRIIIB has been reported to mediate signaling events including actin polymerization (168), and $[Ca^{2+}]_i$ flux (169). Since FcγRIIIB has no cytoplasmic or transmembrane domain, the possibility that FcγRIIIB signals to neutrophils through FcγRII has been suggested. 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 (170). Similar observations have been made

concluding that FcγRIIIB signaling was modulated by FcγRII (171). Other evidence for interaction between neutrophil FcγRIIIB and FcγRII is the enhanced phagocytosis of anti-FcγRII Fab-coated erythrocytes following crosslinking of FcγRIIIB (168). The mechanisms of FcγRIIIB-FcγRIIA interaction are not well understood. Immunoprecipitates of GPI-anchored proteins are reported to co-precipitate Src-family kinases (172). This however, may be due to hydrophobic interactions between the myristoyl group on the kinases and the lipid moiety of the GPI anchor.

Anti-FcγR autoantibodies in autoimmune diseases

Mononuclear phagocyte system (MPS) function, defined by clearance of IgG-coated autologous erythrocytes, is often impaired in patients with different autoimmune diseases including both systemic lupus erythematosus (173) and organ-specific syndromes (174,175). A similar defect is observed in mouse strains prone to lupus-like disease (176). The cause for this dysfunction is not completely understood. The rate of Fc receptor clearance does have a genetic factor since disease-free individuals, who share the same class II major histocompatibility alloantigens also have prolonged *in vivo* clearance (177). However, the variation of T½ values with disease activity, observed in systemic lupus erythematosus (178,179), suggests an additional acquired component resulting in impaired immune complex clearance. Anti-FcγR autoantibodies offer a possible explanation for the inhibition of FcγR-mediated clearance.

Anti-FcγR Ig was reported in SLE and autoimmune neutropenia (5,36,180). The prevalence of anti-FcγR autoantibodies was surveyed in serum samples collected

from different mouse stains prone to autoimmune disease using an ELISA in which soluble denatured mouse FγRII served as the capture reagent (50). This study demonstrated the presence of predominantly IgM anti-FcγR autoantibodies in several strains including NZB, NZB/NZW F_1, MRL, viable motheaten and TSK. The amount of circulating antibody increased with age. Monoclonal antibodies with similar specificity could be also generated from unimmunized NZB, TSK and motheaten mice. Both the mAbs specific for FcγR and affinity purified naturally occurring anti-FcγR Ig bound to murine FcγR-expressing cells and inhibited the binding of immune complexes. Male BXSB mice, which have a severe lupus-like syndrome but normal macrophage FcγR function, did not have any circulating anti-FcγR autoantibody (4). The hypothesis that the paralysis of FcγR function in autoimmune disorders is due to anti-FcγR autoantibodies is further supported by the dramatic inhibitory effect of the anti-FcγRIII mAb 3G8 on the clearance of IgG-sensitized autologous red blood cells in chimpanzees (181).

The mouse IgM anti-FcγR mAbs generated from TSK mice also bind to human neutrophils. The IgM anti-FcγR mAbs were specific for huFcγRIII. MAb binding was inhibited by the anti-FcγRIII mAb 3G8 and the mAbs did not bind to cells transfected with human FcγRIIa cDNA. Incubation of neutrophils with the IgM anti-FcγR monoclonal antibodies resulted in release of hydrolases (alkaline phosphatase, β-glucuronidase and elastase) from both azurophil and specific granules of the cells. The degranulation process was accompanied by dramatic morphological changes (170).

The ELISA using truncated recombinant mouse FcγRII was used to determine

the percentage of positive sera from patients with SLE (24%), progressive systemic sclerosis (40%) and Sjögren's syndrome (11.6%). Both IgG and IgM anti-FcγR antibodies were purified by affinity chromatography on a murine FcγRII affinity column. From the patterns of reactivity of the affinity-purified antibody to neutrophils, monocytes, and IFN-γ treated neutrophils, it was apparent that anti-FcγR Ig isolated from human serum samples could be directed against either FcγRI, FcγRII, or FcγRIII (182). From a patient with end-stage progressive systemic sclerosis generation an EBV-transformed lymphoblastoid cell line was isolated, which secreted an IgG_2 anti-FcγRIII autoantibody that bound to human neutrophils and triggered the release of β-glucuronidase, aryl sulfatase and alkaline phosphatase (183).

A more extended series of sera has now been typed for class specificity of reactivity with recombinant secreted human FcγRII and FcγRIII. Sera from patients with rheumatoid arthritis and systemic lupus erythematosus had exclusively IgM autoantibody reactive with FcγRII and/or FcγRIII. In contrast, sera from patients with primary Raynaud's syndrome showed predominantly IgG reactivity with FcγRIII, and sera from patients with progressive systemic sclerosis displayed both IgG and IgM reactivity towards FcγRII and FcγRIII. Surprisingly, over 50% of sera from patients with osteoarthritis had IgG antibody directed against FcγRII (184). The role of anti-FcγR autoantibodies in the pathophysiology of the autoimmune diseases needs further evaluation.

II. Early Events of Human FcγRIIA Signaling in Transfected Mouse Macrophages

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 signaling events following crosslinking of B and T cell antigen receptors, FcεRI, and FcγRs share common elements (11,12). In each, signaling is initiated by receptor crosslinking by antigen or immune complexes. Subsequent events include phosphorylation on tyrosine (99,185-188), activation of phospholipase C-γ1 (147), and $[Ca^{2+}]_i$ flux (88). There is a conserved tyrosine activation motif with a dyad Y-X-X-L sequence present in Igα, Igβ, FcεRIγ, and CD3ε and ζ (see (12) for review) (Fig. 1A). Site-directed mutagenesis studies of CD3ζ suggest that the two tyrosines in the conserved motif are crucial (189). A related motif, in which the Y-X-X-L sequences are separated by an additional 5 residues, is found in FcγRIIA (Fig. 1A).

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 (190). Previously, we analyzed the structural requirements for these activities by expressing FcγRIIA and truncated FcγRIIA proteins in fibroblast (CHO) and murine macrophage ($P388D_1$) cell lines

(149). The macrophage-based FcγRIIA expression system we have used permits a more physiologically relevant analysis of structure-function relationships and signal transduction than other systems, as this system utilizes a cell line in which FcγRs are normally functional. In these studies, human FcγRIIA was specifically activated using mAb IV.3 Fab (anti-human FcγRII) and an anti-mouse IgG $F(ab')_2$ reagent. This protocol was used to avoid activation of endogenous murine FcγR, and mimics the binding and phagocytosis of soluble immune complexes. We previously demonstrated that CHO cells lack the signaling apparatus required for either FγRIIA-mediated phagocytosis or $[Ca^{2+}]_i$ flux and identified a 31 residue cytoplasmic region (Arg^{234}-Asp^{264}) required for phagocytosis in $P388D_1$ cells. Surprisingly, deletion of the carboxyl-terminal 17 residues Lys^{265}-Asp^{281} (Δ264) (which includes the distal Y-X-X-L motif) ablated the $[Ca^{2+}]_i$ flux while preserving the ability of the receptor to mediate internalization of complexes in $P388D_1$ cells.

Other studies of FcγR transfectants in T cell and fibroblast cell lines have also suggested that these cytoplasmic domains are important in signal transduction. We have now examined by site-directed mutagenesis the importance of specific residues between Arg^{234}-Asp^{264} and the COOH-terminal Y^{268}-X-X-L for signal transduction. In addition to measuring phagocytosis and $[Ca^{2+}]_i$ flux, we compared tyrosine phosphorylation events following crosslinking of wild type (wt) and mutant FcγRIIA expressed in the mouse macrophage cell line.

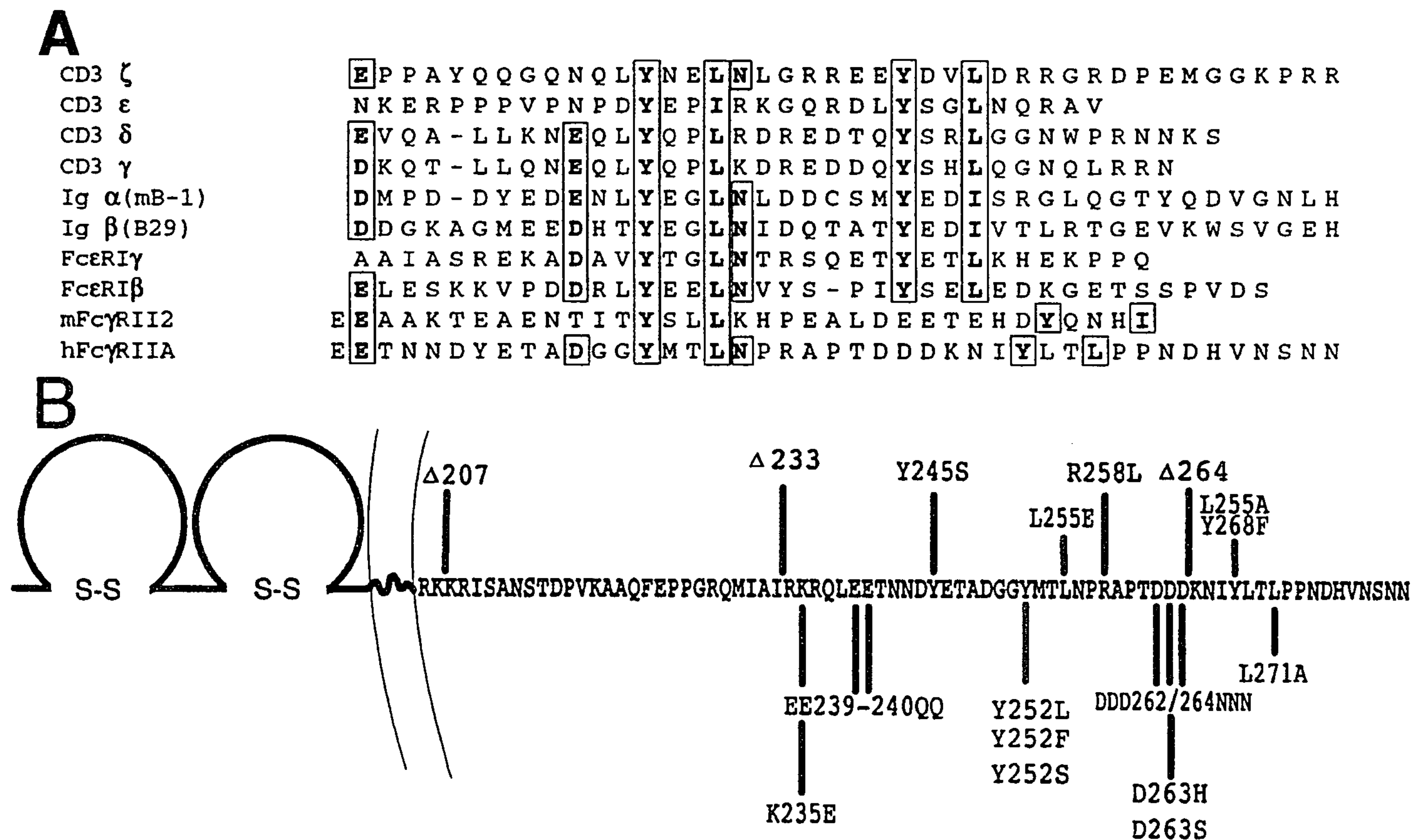

Figure 1. (A) Alignment of cytoplasmic sequences of human FcγRIIA and the tyrosine activation motif. (B) Human FcγRIIA mutations. The sequence of the wild-type FcγRIIA cytoplasmic tail is shown in the single-letter amino acid code. Four truncated molecules, Δ169, Δ207, Δ233, and Δ264 are indicated. Thirteen different amino acid substitution mutants are also indicated. For example, Y252F denotes a $Tyr^{252} \rightarrow Phe$ mutation.

Results

Phagocytosis and calcium flux in $P388D_1$ cells expressing FcγRIIA mutants

The cytoplasmic domain of FcγRIIA has a modified tyrosine activation motif, with 15 amino acids between the Y-X-X-L motifs instead of 10 in the consensus sequence (Fig. 1A). Amino acid substitutions of some of the conserved residues in the cytoplasmic domain of FcγRIIA were introduced by site-directed mutagenesis (191) (Fig. 1B). Internalization kinetics of immune complexes mediated by the FcγRIIA point mutants and the Δ264 truncation mutant were indistinguishable from wt with the exception of the Tyr^{252}→Phe (Y252F), Leu^{255}→Glu (L255E), and Leu^{255}→Ala-Tyr^{268}→Phe (L255A-Y268F) mutants (Fig. 2A), which internalized the little of the surface complexes in a largely temperature insensitive manner (6% at 37°C; 4% at 23°C, 2 μg anti-mouse IgG $F(ab')_2$/ml, 15 min time point). In contrast, internalization of immune complexes by wt-FcγRIIA under identical conditions at 37°C reached a plateau of 80-90% by 15 min, and was inhibited 80% at 23°C (data not shown). The degree of internalization observed for the Y252F, L255E, and L255A-Y268F mutants was dependent on the concentration of goat anti-mouse IgG $F(ab')_2$. At low secondary antibody concentration (2 μg anti-mouse IgG $F(ab')_2$/ml) there was essentially no internalization by the Y252F, L255E, and L255A-Y268F mutants (Fig. 2A), while the wt FcγRIIA efficiently phagocytosed complexes. However, at 10-fold higher secondary antibody concentration (Fig. 2B), there is substantial internalization of the crosslinked complexes by the Y252F, L255E, and L255A-Y268F mutants. This is in contrast to the dose response seen with another mutant FcγRIIA, Δ233, which has

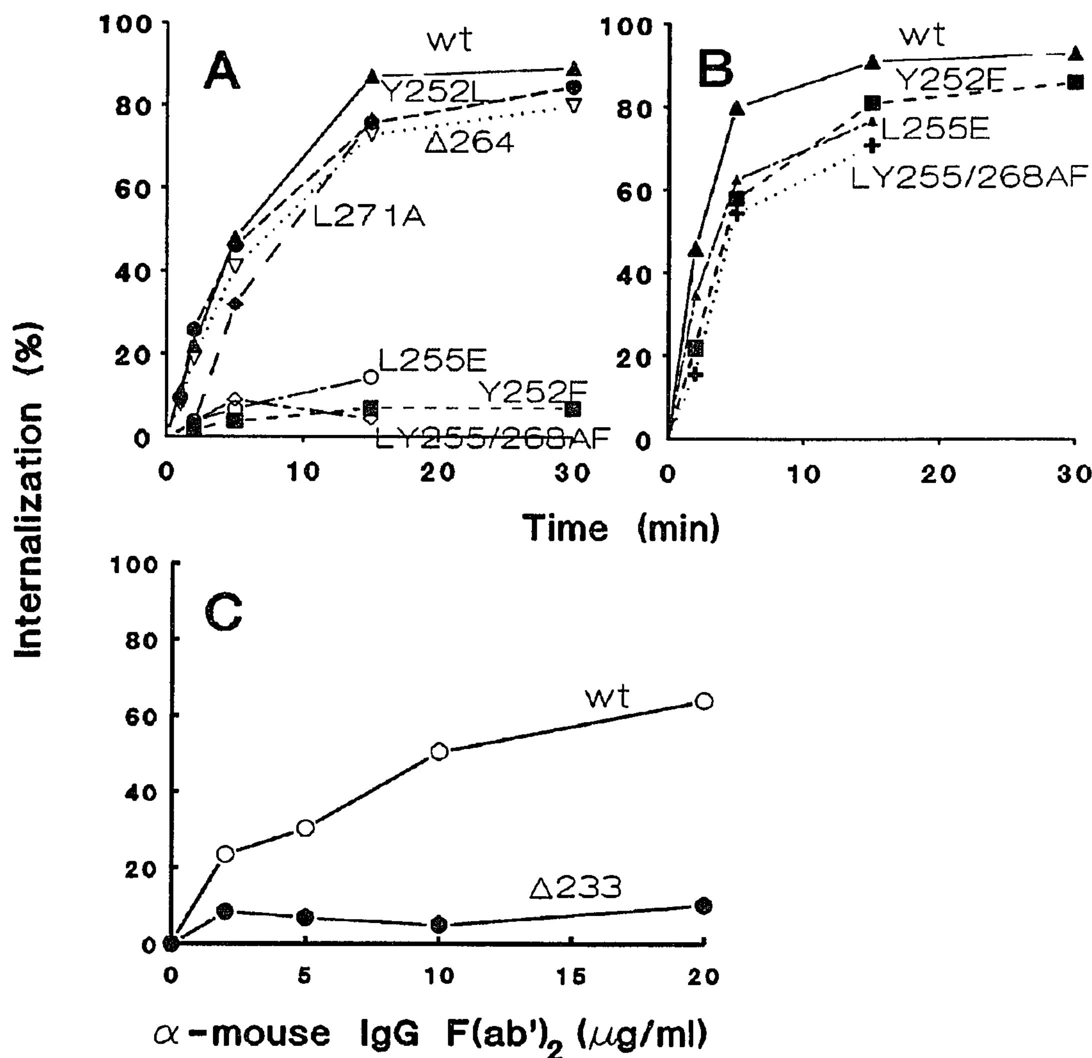

Figure 2. Time course of internalization mediated by wild-type and mutant FcγRIIA. FcγRIIA molecules were decorated with IV.3 Fab and crosslinked with goat anti-mouse IgG $F(ab')_2$ at 4°C. After incubation at 37°C the percentage of internalized complexes was determined as described in Materials and Methods. The concentration of goat anti-mouse IgG $F(ab')_2$ is 2 μg/ml in (A) and 20 μg/ml in (B). In (C) the internalization of complexes, measured at a 5 min interval at 37°C, is shown as a function of goat anti-mouse IgG $F(ab')_2$ concentration for wt FcγRIIA and the Δ233 mutant.

both Y-X-X-L motifs deleted, and which exhibits the same low internalization of immune complexes at 2 and 20 μg/ml of anti-mouse IgG F(ab')$_2$ (Fig 2C). We conclude that the Y252F, L255E, and L255A-Y268F mutants, while severely crippled, are not totally inactivated for internalization of surface bound complexes. Unexpectedly, the Y252L and Y252S mutants internalized complexes as well as wt FcγRIIA, even at low secondary antibody concentrations. Internalization of endogenously expressed murine FcγRII/III complexes by the Y252F transfectant was the same as that observed in P388D$_1$ cells transfected with wt FcγRIIA (data not shown).

To measure $[Ca^{2+}]_i$ flux in wt and mutant FcγRIIA transfectants, cells were loaded with indo-1-AM, stimulated by crosslinking bound IV.3 Fab with 35 μg/ml of anti-mouse IgG F(ab')$_2$, and ratio fluorescence values were determined (192). A high concentration of secondary antibody is necessary to synchronize cell activation for detection transient $[Ca^{2+}]_i$ flux (169). After stimulating wt FcγRIIA there was a prompt elevation of $[Ca^{2+}]_i$ to a mean increase in $[Ca^{2+}]_i$ of 213 nM over a baseline of about 100 nM. After stimulating the Y252F (Fig. 3b), L255E, L255A-Y268F, and L271A (Fig. 3d, e, f) mutants there was no $[Ca^{2+}]_i$ flux, although Y252S and Y252L (Fig. 3c) mutants again responded with increased $[Ca^{2+}]_i$, as did the other point mutants.

Although the internalization of crosslinked FcγRIIA complexes is independent of $[Ca^{2+}]_i$ flux, phagocytosis of erythrocytes coupled with IV.3 Fab (E-IV.3) requires $[Ca^{2+}]_i$ flux and is inhibited by buffering intracellular Ca^{2+} with BAPTA (149).

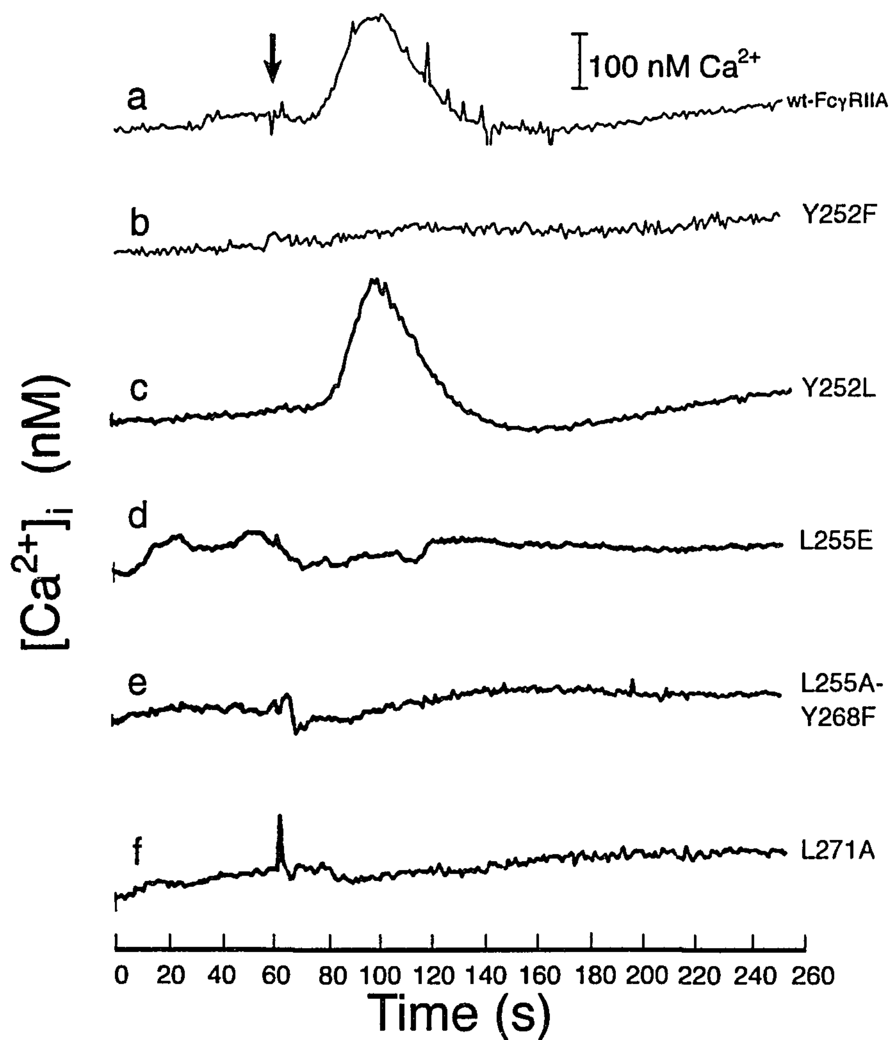

Figure 3. Intracellular calcium increase stimulated by crosslinking FcγRIIA. Suspensions of transfected cells were incubated with indo-1-AM. After incubation with mAb IV.3 Fab, the cells were transferred to a fluorimeter. At 60 sec (↓), goat anti-mouse IgG $F(ab')_2$ was added to initiate receptor crosslinking. Changes in $[Ca^{2+}]_i$ were detected as changes in the Indo-1 405/490 nm fluorescence ratio. The data have been converted to $[Ca^{2+}]_i$ as described (192).

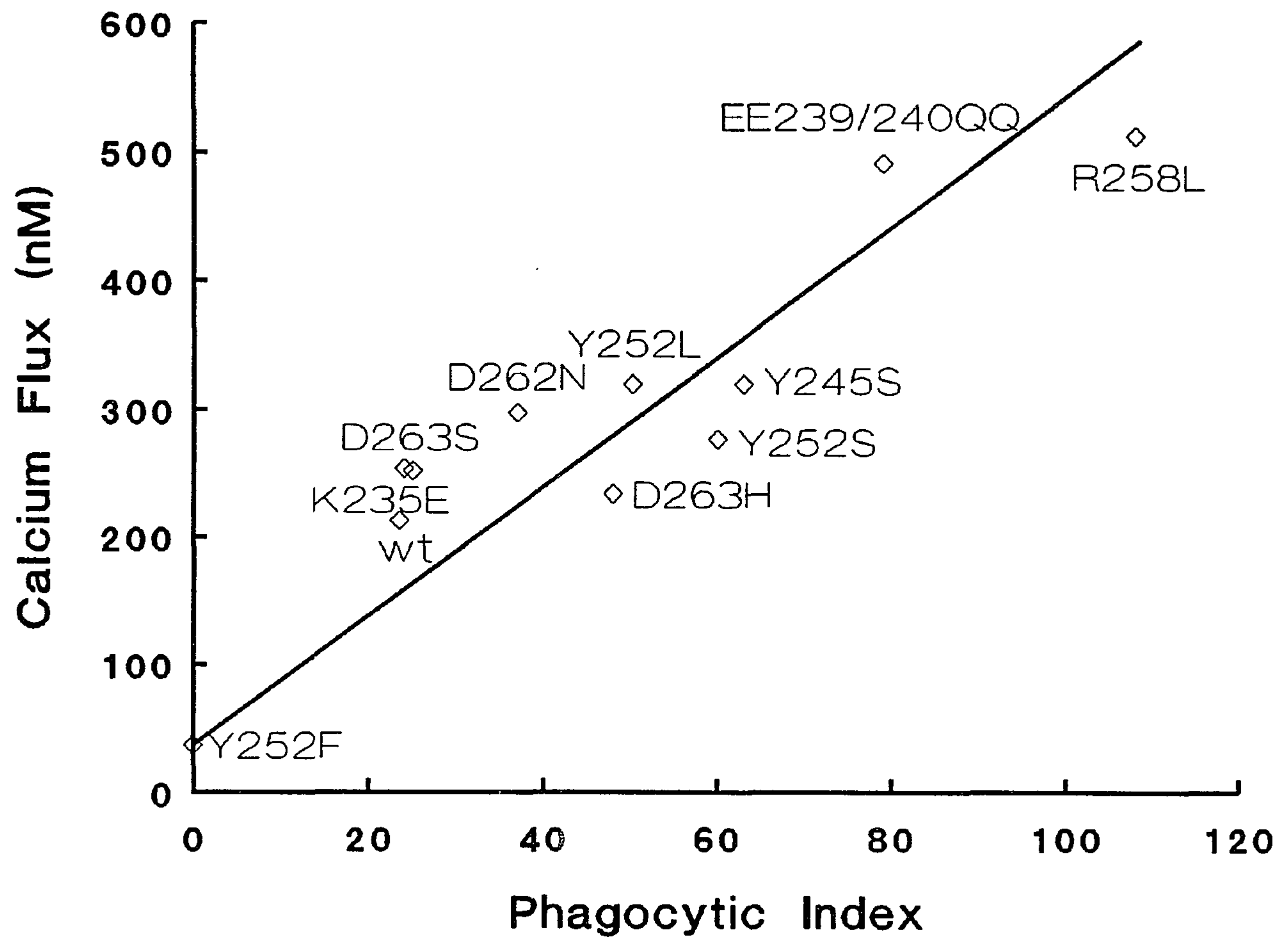

Figure 4. Correlation between $[Ca^{2+}]_i$ flux and phagocytic index for point mutants of FcγRIIA. The phagocytic index is defined as the number of ingested erythrocytes per 100 cells.

Extending these observations, we find that the phagocytic index for E-IV.3 of the different site-directed mutants is correlated with the magnitude of $[Ca^{2+}]_i$ flux triggered by receptor crosslinking (Fig. 4).

Protein tyrosine phosphorylation upon crosslinking FcγRIIA

The kinetics of protein tyrosine phosphorylation in response to crosslinking the wt FcγRIIA were examined. As in the $[Ca^{2+}]_i$ flux experiments, the cells were triggered using anti-mouse IgG $F(ab')_2$ at high concentration (40 μg/ml) to synchronize cell activation, since phosphorylation events are often transitory. Multiple bands of increased intensity were detected in post-nuclear detergent lysates by immunoblot analysis using the anti-phosphotyrosine mAb 4G10 (Fig. 5). The apparent molecular weights (M_r) of bands are indicated in Fig. 5A. Most bands exhibiting increased tyrosine phosphorylation are apparent by 30 sec. The extent of tyrosine-phosphorylation was transient with maximal intensity between 1-2 min returning to baseline levels by 5-10 min. The earliest major band observed (at 15 sec) had a M_r of 72,000 M_r.

No stimulation of tyrosine phosphorylation (at 1 min) was observed in the control $P388D_1$ cell line, PC1. Additionally, no induction of tyrosine phosphorylation was detected at 1 min in a CHO cell line expressing wt FcγRIIA (data not shown). 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 (data not shown).

The rapidly phosphorylated species of 72,000 M_r is the same size as $p72^{syk}$ (also called PTK72) (193), a Syk-family kinase activated by ligation of the B cell antigen receptor (194), the mast and basophil FcϵRI (120), and platelets following activation with wheat germ agglutinin (195). ZAP-70, a kinase activated rapidly upon activation of the TCR (111,196), is closely related to $p72^{syk}$. Immunoprecipitation of lysates from $P388D_1$ after crosslinking of transfected FcγRIIA with an anti-$p72^{syk}$ followed by immunoblotting for phosphotyrosine confirmed the identity of the 72,000 M_r phosphorylated protein as $p72^{syk}$ (Fig. 6).

Protein tyrosine phosphorylation in FcγRIIA mutants

The protein tyrosine phosphorylation patterns stimulated by crosslinking all point and truncation mutants were examined. The nine point mutants with wt function for phagocytosis and $[Ca^{2+}]_i$ flux exhibited essentially wt tyrosine phosphorylation patterns and kinetics, whereas no induction was seen for two completely nonfunctional truncations, Δ233 (Fig. 7) and Δ207 (149) (data not shown). The kinetics of the Δ264 phosphorylation response were normal, with maximal intensities reached by 1-2 min and dephosphorylation occurring by 5-10 min. However, following activation, cells expressing the Δ264 truncation mutant (which cannot flux $[Ca^{2+}]_i$) lacked a subset of the phosphotyrosine-containing proteins including bands of M_r 140,000, 72,000, and 52,000 (Fig 5B) seen in cells transfected with wt FcγRIIA. Immunoprecipitation with anti-$p72^{syk}$ showed the absence of tyrosine-phosphorylation of $p72^{syk}$ following

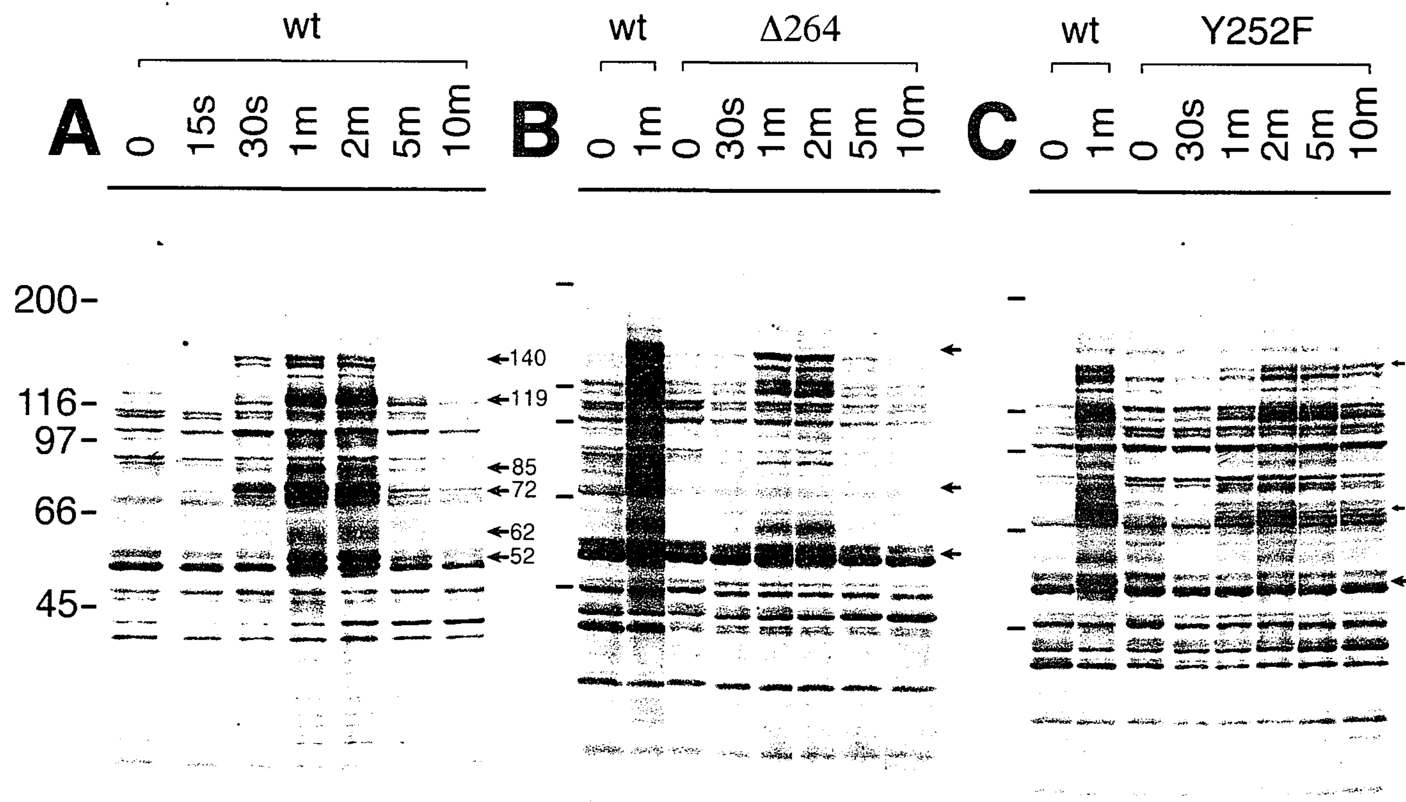

Figure 5. Time course of protein tyrosine phosphorylation following FcγRIIA crosslinking. (A), wt FcγRIIA expressed in $P388D_1$; (B), Δ264 truncation ; (C), Y252F mutation.

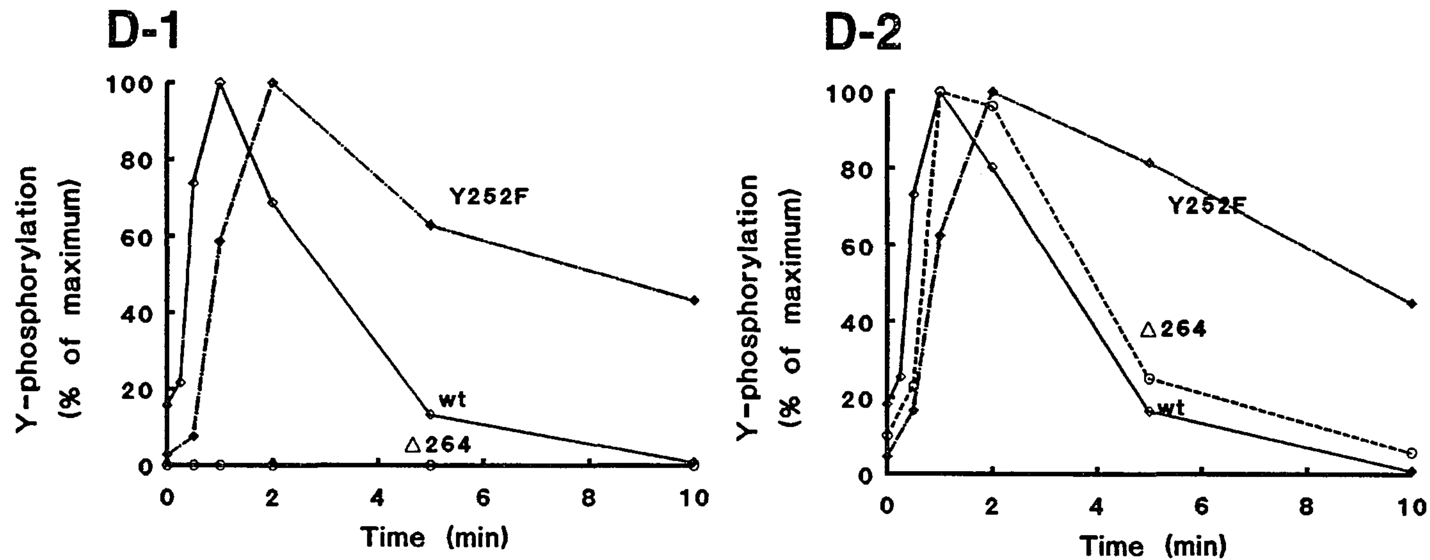

Figure 5. (D) Densitometry of phosphorylated bands induced by crosslinking of wt and mutant FcγRIIA molecules. The densities of the 140,000 M_r band of wt, Y252F and Δ264 mutants (D-1), and the 137,000 M_r band seen in the wt, Y252F and Δ264 (D-2) mutants were quantified and then normalized by dividing by the density of the constitutively phosphorylated bands at 45,000 M_r. The maximum response for each band was defined as 100% phosphorylation to facilitate comparison.

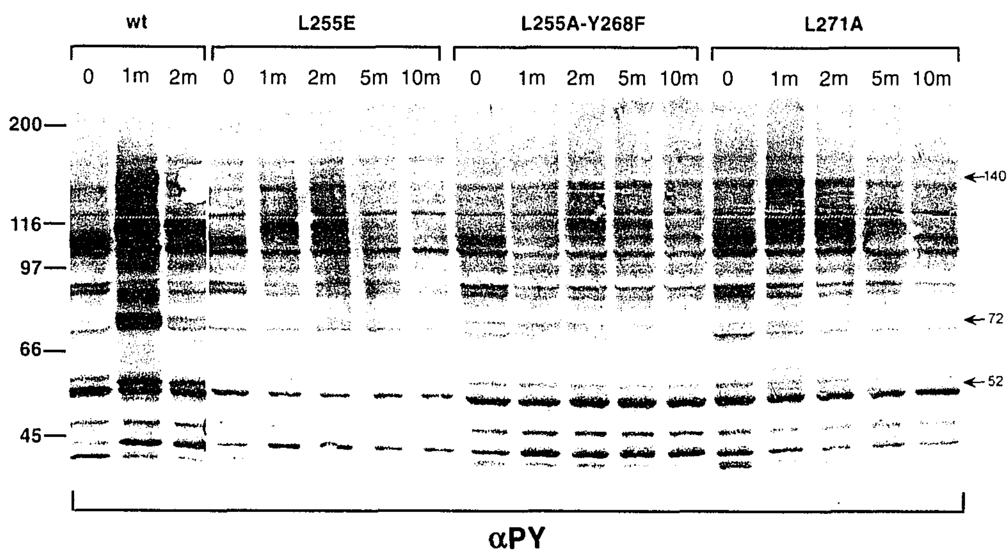

Figure 5. (E) Time course of protein tyrosine phosphorylation by crosslinking of L255E, L255A-Y268F, and L271A FcγRIIA mutants.

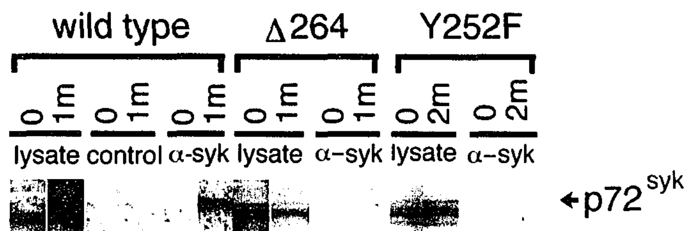

Figure 6. Tyrosine-phosphorylation of p72^{syk} in response to FcγRIIA crosslinking. Lysates from controls or cells in which FcγRIIA was crosslinked for 1 min were immunoprecipitated with rabbit anti-p72^{syk}, subjected to SDS-PAGE, transferred to nitrocellulose, and probed for phosphotyrosine with mAb 4G10.

activation of Δ264 cells (Fig. 6). The Y252F point mutant clearly lacked only the major 52,000 M_r protein, although the relative intensities of others, including p72^{syk}, were substantially less (Fig. 5C, 6). 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. 5D), compared to near total dephosphorylation by the P388D$_1$ line expressing wt FcγRIIA. The phosphorylation and dephosphorylation responses of L255E and L255A-Y268F were similar with those of Y252F whereas L271A had the same responses as wt (Fig. 5E). The L255A-Y268F mutant clearly lacked the tyrosine-phosphorylated 52,000 M_r protein though the relative intensity of this protein was substantially less in the L271A and L255E mutants. L255E, L255A-Y268F, and L271A mutants lacked the tyrosine-phosphorylated 140,000 M_r protein whereas the relative intensity of p72^{syk} was less in these three mutants (Fig. 5E).

Nine of 13 point mutants, including Y252L, Y252S, and Y245S, presented essentially a wt phenotype for $[Ca^{2+}]_i$ flux, internalization, phagocytosis, and phosphorylation on tyrosine residues (Table 3). 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. Therefore, we confirmed the presence of the mutations by sequencing the genomic DNA of these transfectants after PCR amplification with appropriate primers (data not shown).

Table 3. Summary of functions mediated by wild-type and mutant FcγRIIA transfected into P388D_1 cells. Results for huFcγIIA mutants Δ264, Δ233, Δ207 are summarized from Odin et al. (149). All full length mutant receptors were tested on at least two different transfected cell clones. Some cell clones were obtained from transfections done at different times.

	Internalization	Phagocytosis	$[Ca^{2+}]_i$ flux	PY
wt (PW16)	+	+	+	+++
Δ264	+	-	-	+*
Δ233	-	-	-	-
Δ207	-	-	-	-
K235E	+	+	+	+++
EE239-240QQ	+	+	+	+++
Y245S	+	+	+	+++
Y252F	-	-	-	++*
Y252L	+	+	+	+++
Y252S	+	+	+	+++
L255E	-	-	-	++*
R258L	+	+	+	+++
D263H	+	+	+	+++
D263S	+	+	+	+++
DDD262/264 NNN	+	+	+	+++
L255A-Y268F	-	nd	-	+ *
L271A	+	nd	-	+*

*: the relative intensity of tyrosine phosphorylation level is low.
nd: not determined.

Tyrosine phosphorylation of FcγRIIA

Phosphorylation on tyrosine of FcγRIIA was analyzed by immunoprecipitation of FcγRIIA from macrophage cell lysates before and after stimulation followed by immunoblotting with mAb 4G10 (Fig. 8). Maximal phosphorylation of the receptor 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 (data not shown). 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. We never observed, in multiple experiments under conditions where there was strong tyrosine phosphorylation of wt FcγRIIA, tyrosine phosphorylation of the Δ264 mutant, which is lacking the COOH-terminal Y-X-X-L. We observed weak tyrosine phosphorylation following activation of the Y252F mutant, which cannot flux Ca^{2+} or phagocytose E-IV.3 Fab and internalize immune complexes only after extensive crosslinking with a high concentration of goat $F(ab')_2$ anti-mouse IgG (Fig. 9).

Pharmacological studies of FcγRIIA signaling

To assess the functional importance of protein tyrosine phosphorylation, $P388D_1$ cells expressing wt FcγRIIA (PW16) were pretreated (10 μM, 20 hr) with the tyrosine kinase inhibitor, herbimycin A (187). This abrogated tyrosine phosphorylation in response to FcγRIIA activation and dramatically reduced the

amount of tyrosine phosphorylated proteins in the cell (Fig. 8B). The almost total lack of staining by mAb 4G10 of lysates from herbimycin-A treated cells also shows that the staining we observe is specific. Both $[Ca^{2+}]_i$ flux (Fig. 7f) and receptor-mediated internalization of complexes (Fig. 8A) were inhibited in a dose-dependent fashion by herbimycin A pretreatment, with complete inhibition occurring at 10 μM herbimycin A. Loading $P388D_1$ cells with BAPTA-AM (197), a calcium chelator, had no effect on internalization of complexes and no effect on protein tyrosine phosphorylation at 1 min (data not shown).

The abnormal kinetics of tyrosine phosphorylation and dephosphorylation in the Y252F mutant suggested that activation of protein tyrosine phosphatase (PTPase) activity may occur following FcγRIIA stimulation. The importance of PTPase activity during FcγRIIA signaling was examined by treatment with the PTPase inhibitors vanadate, pervanadate, and phenylarsine oxide. Pretreatment of $P388D_1$ cells expressing wt FcγRIIA with vanadate (400 μM, 4 hrs) had no effect on the FcγRIIA-mediated tyrosine phosphorylation pattern at 1 min, and, as expected, no effect on internalization of complexes (data not shown). However, vanadate may not be sufficiently permeable in the $P388D_1$ line to be effective. Short preincubation with low concentrations of pervanadate resulted in a dose-dependent elevation of the basal level of phosphotyrosine, inhibition of tyrosine kinase activity induced by stimulating the transfected FcγRIIA, and inhibition of internalization of complexes (Fig. 8B, C). Phenylarsine oxide (PAO), which reacts with closely spaced sulfhydryl groups (198), is a potent inhibitor of PTPases (199,200). PAO pretreatment of PW16 cells, like

pervanadate, stimulated basal tyrosine phosphorylation, but broadly inhibited FcγRIIA-induced tyrosine phosphorylation (Fig. 8B). $[Ca^{2+}]_i$ flux and receptor-mediated internalization of complexes were inhibited as well (Fig. 8C). The $[Ca^{2+}]_i$ flux was more sensitive to inhibition by PAO (80% inhibition at 5 μM PAO), than was internalization (20 μM PAO was required for 50% inhibition of internalization of complexes at a 5 min time point). However, at later time points, even at 40 μM PAO, the normal patterns of increased tyrosine phosphorylation appeared accompanied by internalization of complexes (data not shown).

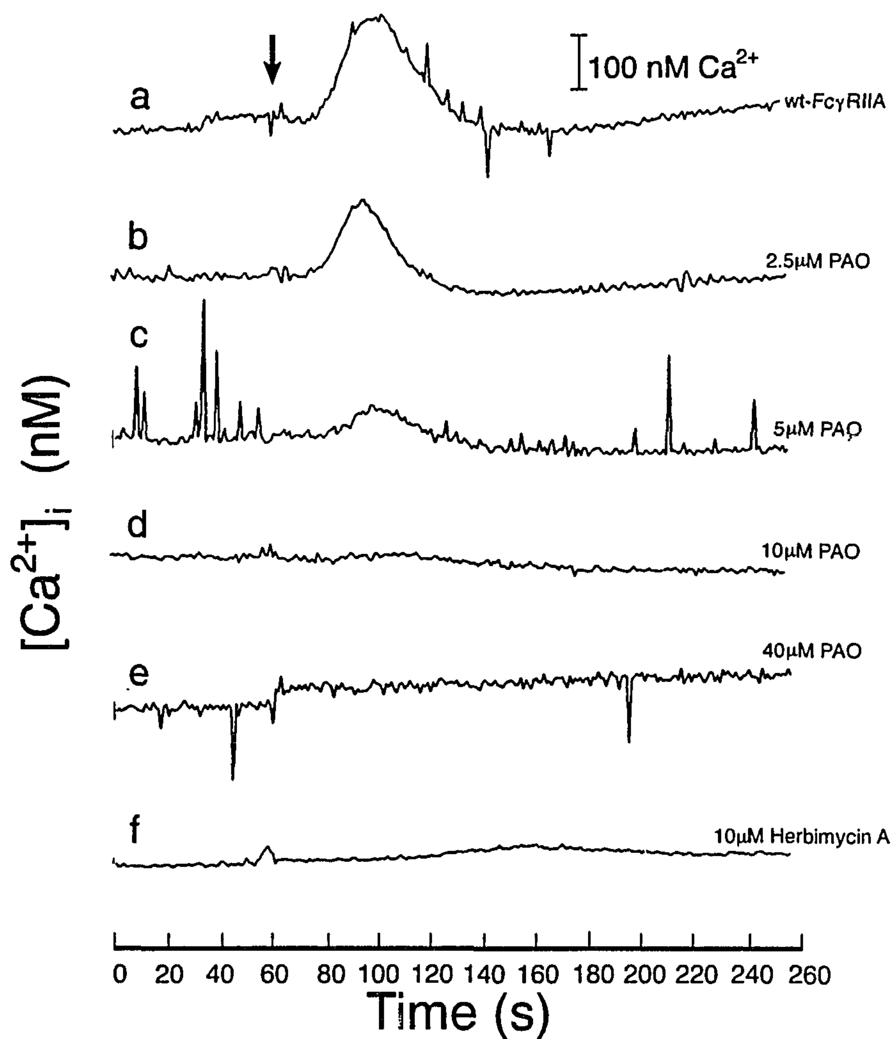

Figure 7. Effect of PAO, and herbimycin A on FcγRIIA-mediated $[Ca^{2+}]_i$ flux. Transfected P388D_1 cells were incubated with the indicated concentration of herbimycin A or PAO as described in Materials and Methods prior to stimulation by receptor crosslinking at 37°C. Changes in $[Ca^{2+}]_i$ were detected as described in Figure 3.

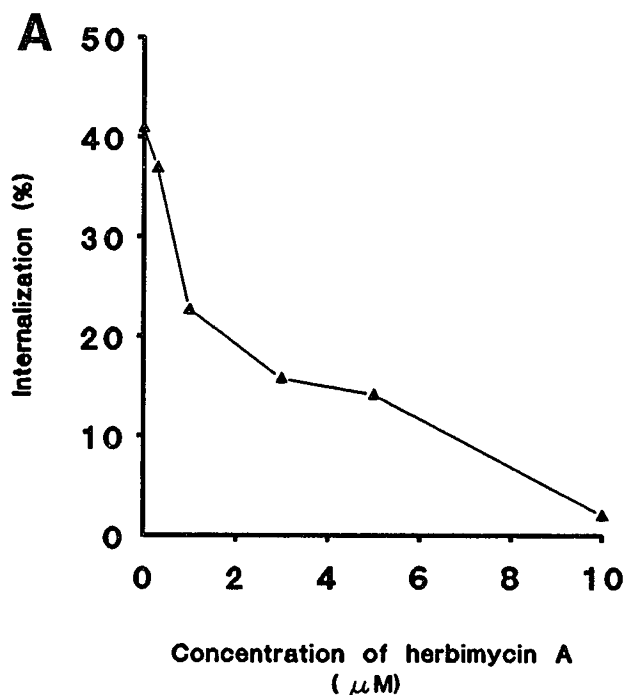

Figure 8. Effect of pervanadate, PAO, and herbimycin A on FcγRIIA-mediated functions. Transfected P388D$_1$ cells were incubated with the indicated concentration of herbimycin A, pervanadate, and PAO as described in Materials and Methods prior to stimulation by receptor crosslinking for 1 min at 37°C. (A)Time course of internalization mediated by wild-type FcγRIIA. FcγRIIA molecules were decorated with IV.3 Fab and crosslinked with goat anti-mouse IgG F(ab')$_2$ at 4°C. After incubation at 37°C at a 5 min interval, the percentage of internalized complexes was determined as described in Materials and Methods. The internalization of complexes is shown as a function of concentration of herbimycin A.

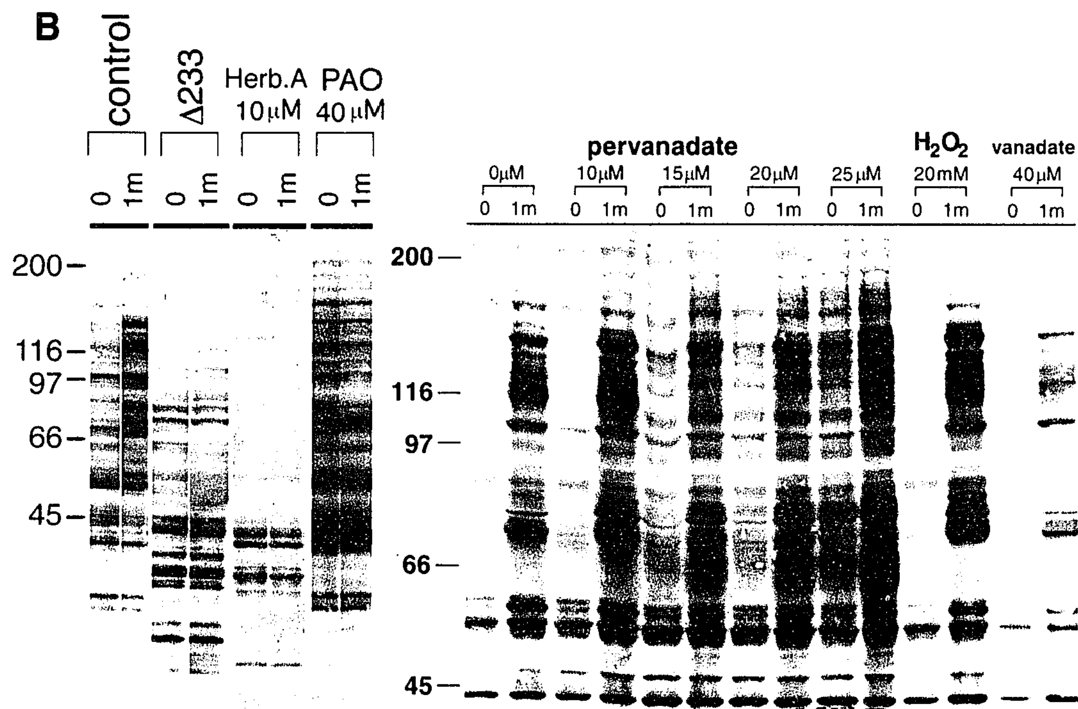

Figure 8. (B) Protein tyrosine phosphorylation before and 1 min after crosslinking FcγRIIA in the presence and absence of 10-25 μM pervanadate, 20 mM H_2O_2, 40 μM vanadate, or 40 μM PAO; effect of preincubation (20 h, 10 μM) with herbimycin A on protein phosphotyrosine; and phosphorylation following crosslinking of the Δ233 FcγRIIA mutant transfected into $P388D_1$ cells.

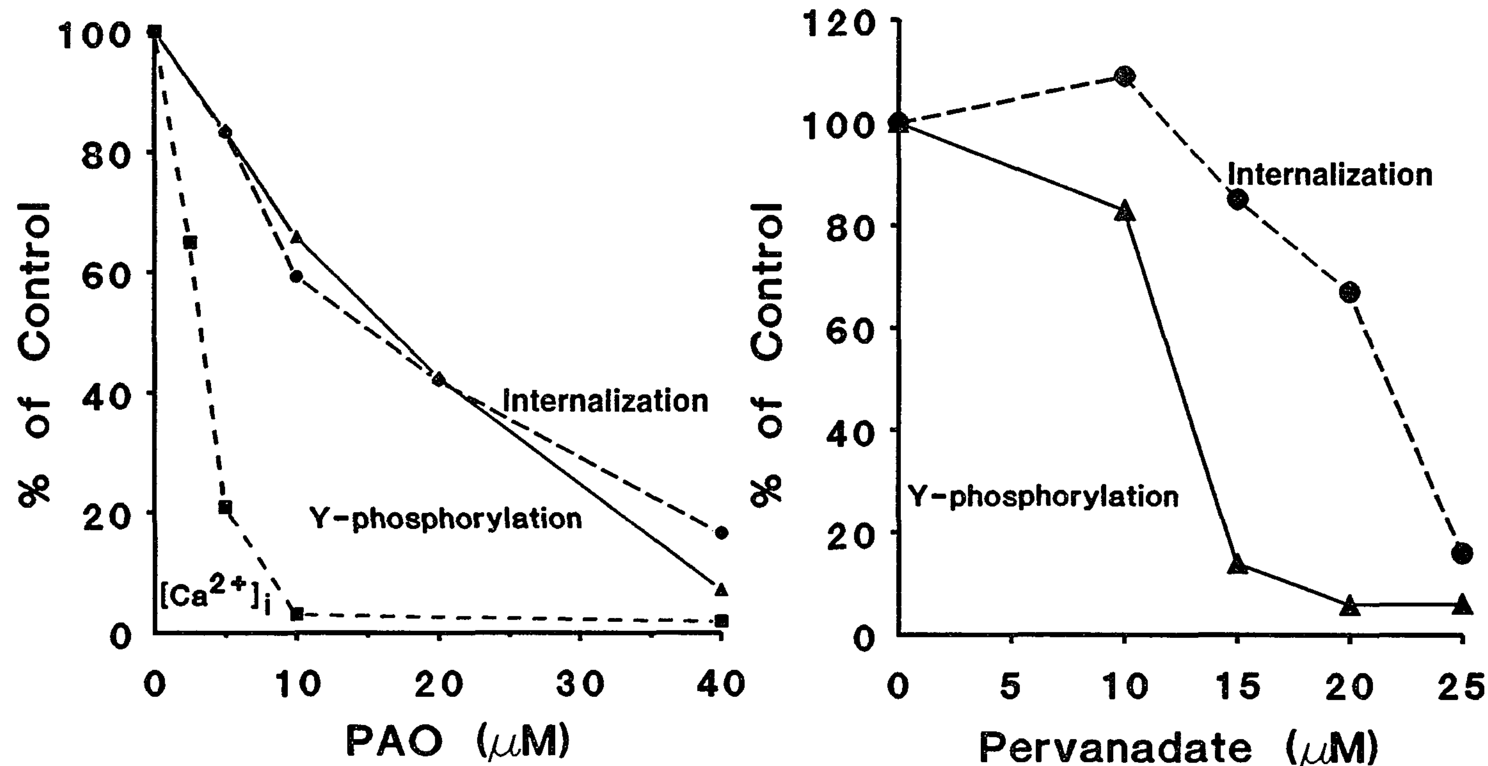

Figure 8. (C) Quantification of phosphorylation, internalization of complexes, and $[Ca^{2+}]_i$ flux as a function of inhibitor concentration. The density of the prominent band at 116 kDa following stimulation was normalized by dividing by the density of the constitutively phosphorylated protein at 45 kDa. The value obtained for stimulated cells without inhibitors was set at 100%.

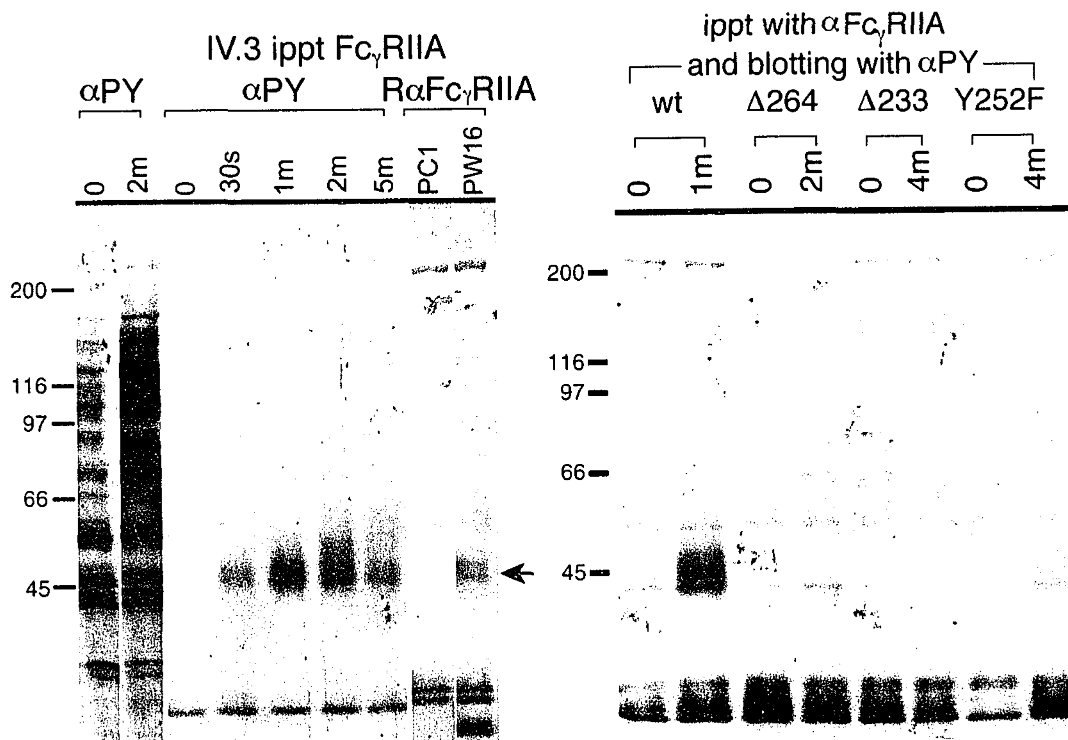

Figure 9. Tyrosine phosphorylation of human FcγRIIA from P388D$_1$ cells expressing FcγRIIA or the Δ233, Δ264, and Y252F mutants following receptor crosslinking. Cell lysates were immunoprecipitated with mAb IV.3, electrophoresed, transferred to nitrocellulose, and probed for phosphotyrosine with mAb 4G10 or for FcγRIIA with a polyclonal rabbit anti-FcγRIIA IgG. PW16 transfectants express wt FcγRIIA, PC1 express no FcγRIIA.

Discussion

The T cell and B cell antigen receptors (TCR and sIg), FcγRIIIA, and FcϵRI are all multi-chain immune recognition receptors (12) in which one or more subunits share the common tyrosine activation motif D/E-X_7-D/E-X_2-Y-X_2-L-X_7-Y-X_2-L in the cytoplasmic domain (11) (Fig. 1). Human FcγRIIA and FcγRIIC also share a related cytoplasmic sequence: E-X_8-D-X_2-Y-X_2-L-X_{12}-Y-X_2-L (22,99). The importance of the tyrosine activation motif has been shown by the ability of several chimeric molecules containing the CD3 ζ cytoplasmic domain to transduce signals to T cells following crosslinking (188,189,201). Indeed, crosslinking of a chimera with an 18 amino acid sequence of ζ that contains one tyrosine activation 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 (189,202).

FcγRIIA, expressed on platelets, monocytes, macrophages, and neutrophils, mediates internalization of immune complexes and is also responsible for the neutrophil superoxide burst (160,162), which is dependent on a flux of $[Ca^{2+}]_i$. Human FcγRIIA, expressed in P388D_1 cells, mediates $[Ca^{2+}]_i$ flux and rapid, temperature-sensitive internalization of complexes (149). Following crosslinking of FcγRIIA expressed in P388D_1 cells, we observed tyrosine phosphorylation of cellular proteins that was maximal at 1-2 minutes and fell to baseline levels by 5-10 min. The pattern and kinetics of tyrosine phosphorylation we observed in P388D_1 cells were very similar to that we observed in U937 cells, activated under similar conditions (data not shown). The rapidity of the dephosphorylation we observed somewhat faster

than has been observed in other systems (99), but is in accord with previous work demonstrating the activation of tyrosine kinases following receptor crosslinking. The importance of the kinase activation was indicated by the inhibition of internalization and $[Ca^{2+}]_i$ flux observed after pretreatment of cells with herbimycin A. Furthermore, nonfunctional FcγRIIA with severe truncations of the cytoplasmic domain expressed in $P388D_1$ cells and wt FcγRIIA expressed in CHO cells did not trigger tyrosine phosphorylation events upon crosslinking.

Unlike human FcγRIIA, neither splice variant of murine FcγRII induces tyrosine phosphorylation events after crosslinking (104,152,203). 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. The human FcγRIIb1 and FcγRIIb2, which also do not trigger $[Ca^{2+}]_i$ flux or cytolysis (151), may be the homologies of murine FcγRII. The ability of murine FcγRIIb2 to localize to coated pits and mediate endocytosis (153,155) 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 (204) and $[Ca^{2+}]_i$ flux (205) is unclear.

In the model used to study internalization, antibody complexes were internalized at very low concentrations of secondary antibody by $P388D_1$ cells expressing wt FcγRIIA. Severely truncated FcγRIIA molecules lacking a tyrosine activation motif were not internalized rapidly even at high crosslinker concentrations. The Y252F, L255E, and L255A-Y268F mutants, although severely crippled, will internalize complexes at high secondary antibody concentrations (Fig. 2). Whether

wild-type and crippled FcγRIIA possess different internalization pathways remains to be determined. Nevertheless, raising the crosslinker concentration does not cause a $[Ca^{2+}]_i$ flux in incapacitated mutants, or tyrosine phosphorylation in the severely truncated mutants Δ233 or Δ207. Our results differ from those obtained after expression of a vaccinia-vector encoding a CD4/FcγRIIA chimera in T cells and monocytes (151). In this study mutation of Tyr^{282} or Tyr^{298} (which correspond to our Tyr^{252} and Tyr^{268}) to Ser resulted in loss of $[Ca^{2+}]_i$ flux and cytolytic activity. We find that the Y252S and Y252L mutants internalize surface complexes and phagocytose E-IV.3 Fab and also flux $[Ca^{2+}]_i$. Furthermore, the deletion of the terminal Y-X-X-L motif or L271A mutant abolishes $[Ca^{2+}]_i$ flux but does not alter the ability to internalize complexes. The rapid and temperature-sensitive internalization of complexes is clearly linked to activation of tyrosine kinases, as mentioned above.

We had shown previously (149) that a mutant of FcγRIIA missing the 17 carboxyl-terminal amino acids, Δ264, though capable of internalization of crosslinked FcγRIIA complexes, failed to mediate either a $[Ca^{2+}]_i$ flux and phagocytosis of 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 (Fig. 4). However, others have found that phagocytosis of EA by murine peritoneal macrophages is not dependent on $[Ca^{2+}]_i$ flux (206,207). These differences may reflect dependence of membrane recycling on $[Ca^{2+}]_i$ flux 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. Alternatively, $[Ca^{2+}]_i$ flux may be necessary but not sufficient to trigger phagocytosis.

Following activation of the Δ264 FcγRIIA mutant, a subset of proteins normally phosphorylated on tyrosine following activation of the wt FcγRIIA is observed (Fig. 5A, B). Since the Δ264 FcγRIIA mutant internalizes immune complexes normally, presumably activation of the internalization pathway is 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. 5A, B, and D). The differences in phosphorylation patterns between wt FcγRIIA and the Δ264 mutant might be due to activation of distinct tyrosine kinases with different specificities. The failure of activation of Δ264 to induce the phosphorylation of $p72^{syk}$ (Fig. 6) argues for this view. Alternatively, one or more of the subset of proteins not phosphorylated on tyrosine in the Δ264 mutant may require direct interaction with epitopes within the COOH-terminal 17 amino acids of the wt receptor in order to be phosphorylated.

$p72^{syk}$ 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 (208) and ZAP-70 has been found to associated with the ζ and ϵ chains of TCR/CD3 following crosslinking of receptor (196,209). Our results indicate clearly that, following crosslinking of FcγRIIA, $p72^{syk}$ is phosphorylated, which is correlated with kinase activation (210). The failure to activate $p72^{syk}$ by the Δ264 mutant argues that

phosphorylation and activation of p72^{syk} may be important for $[Ca^{2+}]_i$ flux but is not necessary for internalization of complexes. The failure to flux $[Ca^{2+}]_i$ and phosphorylate M_r 72,000 protein by the L255E and L271A mutants may confirm this deduction.

The residues we chose to examine in detail were between Arg^{234} to Asp^{264}, a region previously identified as important for internalization of immune complexes (149). Mutation of the acidic clusters at either end of the Arg^{234} to Asp^{264} sequence was without dramatic effect. The importance of the first Y^{252}-X-X-L repeat in the signaling process is shown by the inability of the Y252F and L255E FcγRIIA mutants to either efficiently internalize complexes or flux $[Ca^{2+}]_i$. However mutation of Tyr^{252} to serine or leucine led to FcγRIIA mutants that were fully functional and indistinguishable from wt, even upon analysis of multiple clones of each transfectant. We propose that the aromatic ring of Tyr or Phe, but not Ser or Leu at position 252 blocks events required for activation of p72^{syk} and, following phosphorylation of Tyr^{252}, the inhibition is removed. However, phosphorylation of Tyr^{252} is not needed for activation of tyrosine kinases after crosslinking of the Δ264 mutant, which is not phosphorylated on tyrosine upon activation (Fig. 9). Although Y252F FcγRIIA was crippled, activating this receptor with high concentration of crosslinker led to tyrosine phosphorylation of most of the proteins seen after activation of the wt receptor, with the exception of a major phosphotyrosine containing protein of 52,000 M_r. However, the rate of phosphorylation was somewhat slower, and the rate of subsequent dephosphorylation was markedly inhibited relative to wt (Fig. 5A, C, D). The L255E

and L255A-Y268F FcγRIIA mutants show a similar phenotype to the Y252F mutant (Fig. 5E). This result suggests that the PTPases responsible for the dephosphorylation are not constitutively active, but are induced as a consequence of crosslinking.

Several studies suggest that the PTPase CD45 is involved in T cell activation. Crosslinking of CD45 with CD4 results in activation of T cells whereas ligation of CD45 and the TCR/CD3 complex results in inhibition of activation (123). T cells mutant in CD45 phosphatase activity do not proliferate upon TCR stimulation (211,212). In contrast, phytohemagglutinin T cell blasts proliferated more extensively in the presence of vanadate (213), an inhibitor of some PTPases including CD45 (199,213). However, preincubation of $P388D_1$ cells expressing human FcγRIIA with a high concentration of vanadate (400 μM, 4 h) had no effect on either internalization of immune complexes or tyrosine phosphorylation patterns. We suspect the lack of effect of vanadate may be due to failure to achieve a high enough internal concentration of the inhibitor. Pervanadate, which penetrates cells more readily, is a potent inhibitor of PTPase and dramatically stimulates tyrosine phosphorylation (214). Pervanadate, at intermediate concentrations, while increasing the general phosphotyrosine background in the immunoblot, decreased both the specific tyrosine-phosphorylation events that resulted from stimulation of FcγRIIA, and the internalization of complexes (Fig. 8A, B). Similar results (Fig. 8A, B) were obtained with a very different inhibitor of PTPases, phenylarsine oxide (PAO), which forms a complex with closely spaced sulfhydryl groups (215,216), but does not inhibit tyrosine kinases such as the insulin receptor kinase (199), Fyn, or Lck (200). Thus,

we suggest that PTPase regulates, and may induce the activation of tyrosine kinase(s) following FcγRIIA activation.

The tyrosine phosphorylation of the FcγRIIA itself is not visible in the total lysate of the cells, even though these cells express 10^6 receptors/cell. However, immunoprecipitation of the receptor after crosslinking showed that the receptor is phosphorylated on tyrosine (Fig. 8). However, we were never able, in repeated attempts, to obtain phosphorylation of the Δ264 FcγRIIA, although crosslinking of this receptor clearly activates tyrosine kinase activity (Fig. 5) and triggers internalization of complexes. Since mutation of Tyr^{252} to either Ser or Leu, or Tyr^{245} to Ser, had no effect on the phenotype of the FcγRIIA response, we suggest that one of the residues phosphorylated upon activation is Tyr^{268}. Yet, Tyr^{252} is a critical residue since mutation to phenylalanine (Y252F) resulted in a functionally crippled receptor. Further site-specific mutations are necessary to fully analyze these events and the relationship between the two Y-X-X-L motifs.

These results strengthen the hypothesis that distinct signaling pathways are activated by FcγRIIA for internalization of complexes and for $[Ca^{2+}]_i$ flux. We have shown that protein tyrosine phosphorylation is a prerequisite for both of these functions. Furthermore, $p72^{syk}$, 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, L255E, L255A-Y268F and L271A. Since Δ264 and L271A internalize complexes normally, this suggests that $p72^{syk}$ plays no role in internalization, but is important for $[Ca^{2+}]_i$ flux.

Materials and Methods

Reagents

MAb IV.3 from ATCC (American Type Culture Collection, Rockville, MD) 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 column and mono Q chromatography. The anti-phosphotyrosine mAb 4G10 was purchased from UBI, 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. Unconjugated and biotinylated goat anti-mouse IgG $F(ab')_2$ were purchased from Jackson Immunoresearch. Herbimycin A (187) was very kindly provided by Dr. Yoshimasa Uehara. PAO was purchased from SIGMA. Bapta-AM and Indo-1 were purchased from Molecular Probes, Inc. (Eugene, OR).

Site-directed mutagenesis

A human FcγRIIA cDNA clone was generously donated by Dr. J. Kochan (Hoffman La-Roche, Nutley, NJ). Mutant FcγRIIA cDNAs (Fig.1) were constructed by oligonucleotide primer-directed site-specific mutagenesis (191), confirmed by sequencing (Sequenase Version 2.0, United States Biochemical Corp., Cleveland, OH), and subcloned into the EcoRI site of the eukaryotic expression vector pcEXV-3 (217), which has an SV40 early gene promoter and polyadenylation signal.

Transfection of $P388D_1$ cells

Transfections of $P388D_1$ cells were performed by a modification of the

calcium phosphate co-precipitation method (218) as described (149). To screen transfectants, cells in suspension (2.5 x 10^6/ml) were incubated with mAb IV.3 Fab (1 μg/ml, 1 hr, 4°C), washed by centrifugation, and stained with FITC-conjugated $F(ab')_2$ goat anti-mouse IgG (20 μg/ml, 1 hr, 4°C). Cells were analyzed on a Coulter Epics Cell Sorter (Coulter Corporation, Opa Locka, FL). Cells analyzed for function were populations obtained from repeated (2x) sorting of the brightest 2% of stained cells, or from limiting dilution cloning. The amount of mutant FcγRIIA expressed was quantified by flow cytometry relative to the PW16 cell line, which expresses 1.2 x 10^6 wt receptors/cell. All transfected $P388D_1$ cells expressed between 1.1 - 2.5 x 10^6 receptors/cell (data not shown). The expression of FcγRIIA in $P388D_1$ cells has been stable for at least 6 months of continuous cell culture.

To verify mutations in cultured $P388D_1$ transfectants, genomic DNA was isolated and the region between Ile^{167} and Asn^{281} was amplified by PCR using appropriate primers. PCR products were purified from agarose gel run in TAE buffer using GENECLEAN II (Bio101 Inc., La Jolla, CA) and were subcloned into M13mp19 plasmid for sequencing.

Measurement of internalization and phagocytosis

Transfected cells in suspension were incubated with mAb IV.3 Fab (1 μg/ml, 30 min, 4°C), washed, and then incubated with goat anti-mouse IgG $F(ab')_2$ (Jackson ImmunoResearch, Inc., West Grove, PA) (2 μg/ml, 20 min, 4°C). To activate endogenous muFcγRII/III on the $P388D_1$ cells, mAb 2.4G2 Fab (47) and rabbit anti-rat IgG $F(ab')_2$ were substituted. The cells were then shifted to 37°C for the

indicated intervals and washed at 4°C prior to labeling with FITC-conjugated rabbit anti-goat IgG $F(ab')_2$ (30 μg/ml, 30 min, 4°C) (or FITC-conjugated goat anti-rabbit IgG $F(ab')_2$ when studying endogenous murine FcγRII/III). Background fluorescence was determined by omitting mAb IV.3 or mAb 2.4G2. The fluorescence of labeled cells maintained at 4°C throughout the experiment was used as the control for 0% internalization. Background cell fluorescence represented 100% internalization. Using a linear conversion scale, the fluorescence levels of cells incubated at 4°C and at 37°C were compared to background cell fluorescence. To calculate the percentage of antibody complexes internalized by labeled cells incubated at 37°C, the following equation was used: 100 x {1-[(comparative fluorescence of 37°C cells-1) ÷ (comparative fluorescence of 4°C cells-1)]}. Thus, receptor internalization is detected as a decrease in crosslinked FcγR complexes present on the cell surface. This decrease is not due to shedding of the complexes (149). The phagocytosis of erythrocytes coated with mAb IV.3 Fab (E-IV.3) (219) was performed as described previously (149).

Measurement of $[Ca^{2+}]_i$

Suspensions of transfected cells (10^7/ml) were incubated with 5 μg/ml of Indo-1-AM (Molecular Probes, Inc., Eugene, OR) for 15 minutes at 37°C. After the cells were washed and resuspended in PBS containing 5 mM KCl and 5 mM glucose, they were incubated with mAb IV.3 Fab (1 μg/ml, 37°C, 5 min), washed, and resuspended in PBS containing 2 mM $MgCl_2$ and 1.2 mM $CaCl_2$ for 5 min. The stirred cell suspension was then transferred to a fluorimeter to determine the resting

fluorescence emission ratio (405 nm/490 nm) during excitation at 355 nm. After 60 seconds, goat anti-mouse IgG $F(ab')_2$ (35 μg/ml final concentration) (Jackson ImmunoResearch, Inc.) was added. The maximal emission ratio was determined by lysing cells in 1% Triton X-100, and the minimal ratio by adding EDTA (40 mM). Indo-1 fluorescence emission ratios were converted to $[Ca^{2+}]_i$ as described previously (192).

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. Pelleted cells (1500 rpm, 3 min, Shandon microcentrifuge) 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 100 μl of DMEM plus Hepes containing 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 ($\sim$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 $Na\text{-}PO_4$, 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). Following electrophoresis of cleared lysates on a 7-17% SDS-polyacrylamide

gel (220), gels were electroblotted onto nitrocellulose. 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 room temperature. The blots were incubated with 1 μg/ml of the anti-phosphotyrosine mAb 4G10 (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 (50). The nitrocellulose blots were digitized, and densities of individual bands were quantified using NIH Image 1.44 software.

Inhibitor studies

Cells were preincubated with herbimycin A (187) (10 μM, 16-20 hr, 37°C), a kind gift of Dr. Yoshimasa Uehara (National Institute of Health, Tokyo, Japan). A preliminary screen showed that $P388D_1$ cells can tolerate 400 μM vanadate for 4 hrs without undue toxicity. In experiments to analyze phosphotyrosine and $[Ca^{2+}]_i$ flux, phenylarsine oxide (5-40 μM) was added for 5 min at 37°C before crosslinking; for immune complex internalization the inhibitors were added for 20 min at 4°C before crosslinking. Pervanadate was generated by incubation of 5 parts of 10 mM orthovanadate with 1 part of 500 mM H_2O_2 in modified Tyrode's solution for 10 min at 23°C prior to use (214). Cells were preincubated with the pervanadate for 30 min at 37°C (for phosphotyrosine analysis) or at 4°C (for internalization) before crosslinking. H_2O_2 alone had no effect on either phosphotyrosine and internalization analysis. In all pharmacologic studies, the agent used for pretreatment was

continuously present during stimulation.

Immunoprecipitation of FcγRIIA and p72^{syk}

To immunoprecipitate FcγRIIA, mock and stimulated cell lysates were obtained as detailed above, except a biotinylated secondary crosslinking antibody was used. This enabled subsequent immunoprecipitation of the biotinylated complexes with streptavidin-conjugated agarose. Following overnight incubation with the streptavidin agarose, immunoprecipitates were washed 5 times in lysis buffer and bound proteins released by boiling in SDS sample buffer. Samples were equally divided onto two electrophoresis gels. One blot was probed for phosphotyrosine and 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 (183). 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 $F(ab')_2$.

Mock and stimulated cell lysates were immunoprecipitated with anti-p72^{syk} elicited by immunization with a peptide corresponding to the COOH-terminal 28 amino acids of the porcine Syk sequence coupled to BSA. After SDS-PAGE and transfer to nitrocellulose, the blot was probed with mAb 4G10.

III. Activation of Downstream Signaling Proteins Followed Crosslinking FcγRIIA: Correlations Among Tyrosine Phosphorylation of Shc, $p72^{syk}$, PLC-γ1, and $[Ca^{2+}]_i$ Flux in FcγRIIA Signaling

Shc (M_r 52,000) is phosphorylated on tyrosine residues upon crosslinking FcγRIIA

Several signaling proteins including Shc (221) and PLC-γ1 (140,222) are physically associated with activated growth factor receptors. Shc, a highly conserved and widely expressed protein, has two initiation codons, encoding two overlapping proteins of 47 and 52 kDa that contain a single C-terminal SH2 domain, an adjacent glycine/proline-rich motif that is homologous to the α1 chain of collagen, but no identifiable catalytic domain. Activated PDGF receptor forms a complex with and phosphorylates Shc. Overexpression of Shc in NIH3T3 cells leads to transformation (221). In Rat-2 cells transformed by the v-*src* or v-*fps*, Shc is highly tyrosine-phosphorylated and tyrosine phosphorylation of Shc is rapidly induced upon activation of temperature-sensitive v-src or v-fps nonreceptor tyrosine kinases (223). In addition, tyrosine phosphorylated Shc forms a complex with a nonphosphorylated 23 kDa polypeptide encoded by the *grb2/sem5* gene (137,224).

In our previous studies, crosslinking of wt FcγRIIA in PW16 cells led to the rapid and transitory phosphorylation on tyrosine of a distinct set of proteins including a protein of 52,000 M_r (Fig. 5A). This tyrosine phosphorylated protein was identified to be Shc by Shen et al. (225). Moreover, crosslinking the FcγRIIA deletion mutants Δ264 (Fig. 5B) and Δ233 did not result in tyrosine phosphorylation of 52,000 M_r

protein (Fig. 7A), and crosslinking of the Y252F, L255E, and L271A FcγRIIA mutants resulted in minimal tyrosine-phosphorylation of 52,000 M_r protein (Fig. 5C & 5E). Immunoprecipitation with anti-Shc sera from the Y252F mutant cell lysates showed the same conclusions that the mutant resulted in a small amount of Shc (M_r 52,000) phosphorylation upon crosslinking(225).

Tyrosine phosphorylation of PLC-γ1 (M_r 140,000) upon FcγRIIA activation

PLC-γ1 is one of the several PLC isoforms that convert phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), leading to the activation of protein kinase C (PKC) and the release of the intracellular stores of Ca^{2+}, respectively. Activation of PLC-γ1 is known to occur through tyrosine phosphorylation without the participation of G proteins (140,222,226,227). Tyrosine phosphorylation is correlated with the activation of PLC-γ1 and association with activated PDGF and EGF receptors (131,228,229). Serine phosphorylation of PLC-γ1 by either cAMP-dependent kinase or PKC may serve to modulate the interaction of the enzyme with tyrosine kinases and phosphatases.

Crosslinking of FcγRIIA in PW16 cells resulted in tyrosine phosphorylation of multiple cellular proteins and mobilization of intracellular calcium. Shen et al. (225) confirmed that the 140,000 M_r protein that is tyrosine phosphorylated after activation of FcγRIIA is PLC-γ1. However, no tyrosine phosphorylation of 140,000 M_r protein was detected in Δ264, Y252F, L255E and L271A FcγRIIA mutants (Fig. 5B, 5C & 5E) and none of which triggers $[Ca^{2+}]_i$ flux. Therefore, no tyrosine phosphorylation

of PLC-γ1 was seen by immunoprecipitating PLCγ-1 from these FcγRIIA mutant cell lysates (225).

Effects of PMA

Since phagocytosis by FcγR in neutrophils is associated with production of IP3 and diacylglycerol, resulting in PKC activation (230), we examined the effects of the PKC agonist phorbol myristate acetate (PMA). A short pretreatment of PW16 cells with PMA enhanced the initial rate, but not the final extent of FcγRIIA-mediated internalization (Fig. 10A). Pretreatment of PW16 cells with 1 μM and 25 μM of the protein kinase inhibitor, H8 (231), which inhibits only PKA at < 5 μM but inhibits both PKC and PKA at 25 μM, had no effect on FcγRIIA-mediated internalization of complexes at either concentration (Table 4). However, pretreatment with PMA decreased the level of $[Ca^{2+}]_i$ flux following FcγRIIA crosslinking in a dose-dependent manner (Fig. 10B).

Effects of cyclosporin A

The immunosuppressant drug cyclosporin A (CsA) has been shown to inhibit signal transduction via the high affinity FcϵRI expressed by basophils and mast cells (232) and the antigen receptors on T and B cells (233). These receptors form multi-protein signal transduction complexes, whose accessory chains contain a conserved cytoplasmic motif also present in FcγRIIA (Fig. 1A). CsA becomes active when complexed to intracellular proteins named immunophilins (cyclophilins), and then this immunosuppressant-receptor complexes function to block phosphatase activity by binding to the biological target, Ca^{2+}/calmodulin-dependent PP2B (calcineurin) (233).

However, preincubation of PW16 cells with CsA (0.5-4 μg/ml, 37°C, 2 hr) had no effect on internalization of immune complexes (Table 4).

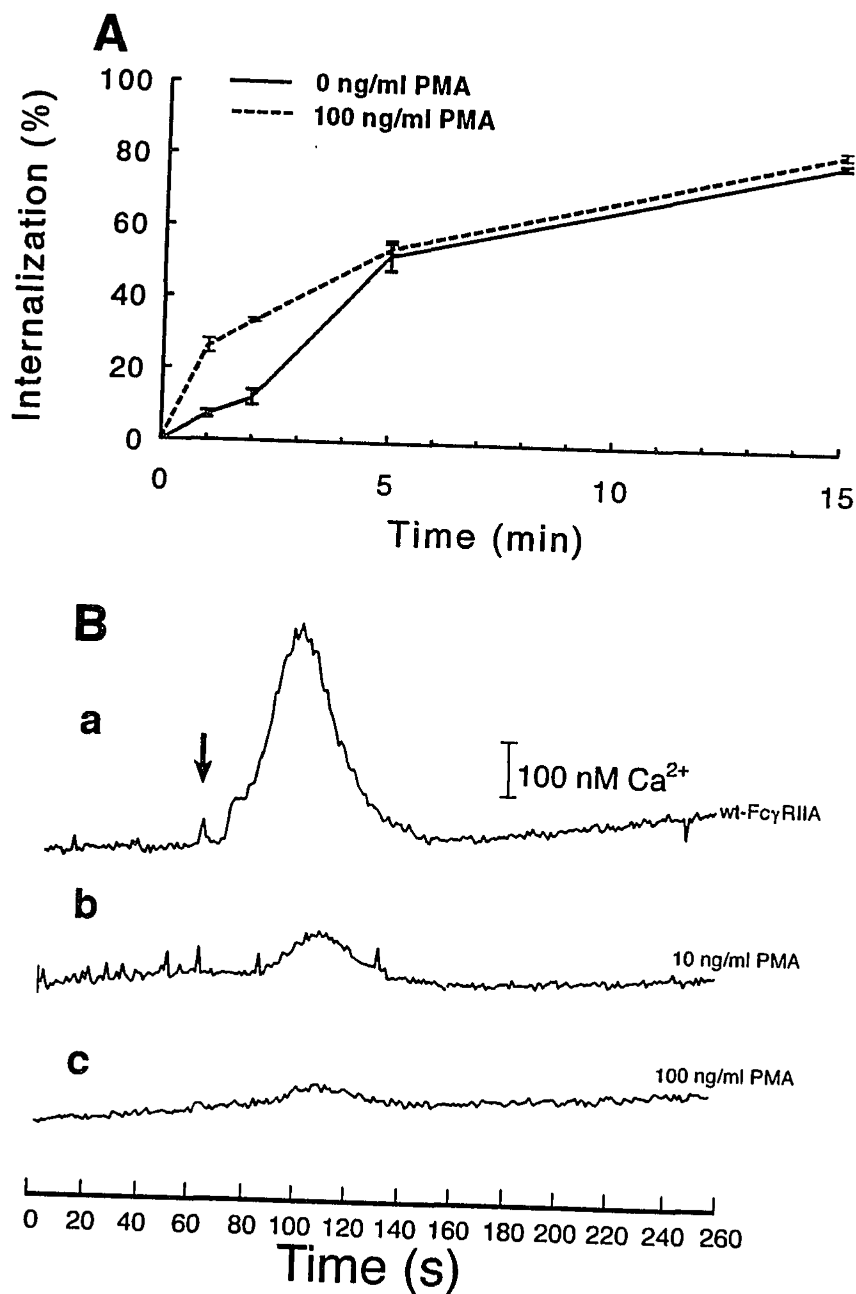

Figure 10. Analysis of PMA treatment on the internalization of receptor complexes and $[Ca^{2+}]_i$ flux following crosslinking of FcγRIIA. (A) Internalization of immune complexes. (B) $[Ca^{2+}]_i$ flux, measured by indo-1 ratio fluorescence.

Table 4. Regulation of FcγRIIA-mediated internalization, $[Ca^{2+}]_i$ flux, and tyrosine-phosphorylation.

Reagents	Internalization	$[Ca^{2+}]_i$ flux	PY
PMA	+	-	+
Calphostin C	same	nd	same
H8	same	nd	nd
Herbimycin A	-	-	-
PAO	-	-	-
Vanadate	same	nd	same
Cyclosporin A	same	nd	nd

The effects of PMA (100 ng/ml), calphostin C (0.5-8 μM), H8 (1 and 25 μM), herbimycin A (10 μM), PAO (2.5-40 μM), vanadate (400 μM), or cyclosporin A (0.5-4 μg/ml) on these FcγRIIA mediated events were determined. nd, not determined; -, inhibited; +, stimulated; same, unchanged

Discussion

We have been interested in dissecting which substrates for cellular kinases are needed for specific effector functions. P388D_1 cells expressing Δ264 FcγRIIA, in which the COOH-terminal Y-X-X-L motif is deleted, can internalize immune complexes but cannot phagocytose large particles nor mediate a $[Ca^{2+}]_i$ flux. Crosslinking Δ264 mutant results in tyrosine phosphorylation of a subset of the proteins seen following activation of the wt FcγRIIA. The Y252F FcγRIIA mutant cannot mediate a $[Ca^{2+}]_i$ flux, and is severely compromised in internalization of immune complexes.

The $[Ca^{2+}]_i$ flux observed in B cells, T cells, and macrophages upon crosslinking of antigen receptors (234,235), FcγRI, and FcγRII (147) is due to activation of PLC-γ1 by tyrosine phosphorylation resulting in the production of diacylglycerol and IP3. PLC-γ1 was rapidly phosphorylated on tyrosine after FcγRIIA activation, accompanied by a corresponding elevation of IP3 that led to $[Ca^{2+}]_i$ flux. However, following activation, there was neither phosphorylation of PLC-γ1 nor $[Ca^{2+}]_i$ flux in either the Δ264 or the Y252F mutants.

We were surprised to observe that pretreatment with PMA resulted in inhibition of $[Ca^{2+}]_i$ flux initiated by FcγRIIA activation. In Jurkat cells, PLC-γ1 tyrosine phosphorylation induced by CD3 crosslinking is inhibited by PMA, as is $[Ca^{2+}]_i$ flux (141). However, we did not observe any decrease in the tyrosine phosphorylation of PLC-γ1 after activation of PW16 cells treated with increasing amounts of PMA relative to controls. Under these conditions, however, there was a

decrease in the production of IP3 in PMA-treated cells (225).

We found that activation by crosslinking of FcγRIIA expressed in P388D$_1$ cells led to rapid and transient tyrosine phosphorylation of 52,000 M_r protein (Shc), thought to be an adaptor that couples tyrosine kinases to downstream targets that lack SH2 domain. Shc is associated with, and is phosphorylated on tyrosine by activated EGF receptor (221), and Shc may be an *in vivo* substrate for the v-Src and v-Fps non-receptor tyrosine kinases in Rat-2 cells transformed by v-*src* or v-*fps* (223). Although PMA had no effect on tyrosine phosphorylation of PLC-γ1, pretreatment with PMA did inhibit the tyrosine phosphorylation of Shc (225).

Activation of protein tyrosine kinases after ligand binding has been shown to be the primary event for signaling by members of the multichain immune recognition receptor family. FcϵRI is reported to activate Lyn and Yes kinases (105), stimulation of FcγRIIIA results in Lck activation (102), and neutrophil FcγRII stimulation activates the Fgr kinase (117). These kinases then activate a second tyrosine kinase, p72^{syk}, which participates in activation of B cells by the antigen receptor (208), RBL-2H3 rat basophilic leukemia cells by the high avidity FcϵRI (120), and platelets activated by lectins (193). A closely related kinase, ZAP-70, is activated in T cells (111). Our previous results confirm recent work demonstrating that p72^{syk} was activated upon crosslinking FcγRIIA (121). We have also found that p72^{syk} was not tyrosine phosphorylated in mutants that failed to trigger $[Ca^{2+}]_i$ flux (see part II for detail). Since the major tyrosine phosphorylated proteins missing in the mutants were PLC-γ1 (M_r 140,000) and Shc (M_r 52,000), the above results argue that p72^{syk} is

responsible for tyrosine phosphorylation of PLC-γ1 and Shc.

The role of the protein kinase C signaling in FcγRIIA activation of macrophages is not well understood. Enhancement of neutrophil phagocytosis by PMA (236) has been reported, and PMA has also been shown to induce the internalization of the TCR/CD3 complex (237), CD4 (238), and the IgE receptor (239), but not surface immunoglobulin on murine B lymphocytes (240). We find that PMA has a slight effect on acceleration of internalization of complexes, but inhibits strongly $[Ca^{2+}]_i$ flux induced by receptor activation. Although PMA had no effect on tyrosine phosphorylation of PLC-γ1 or $p72^{syk}$ induced by FcγRIIA activation, it did inhibit Shc phosphorylation to roughly the same extent as $[Ca^{2+}]_i$ flux was inhibited (225). Our results differ from previous studies that found PMA treatment to result in a dose- and time-dependent reduction of PLC-γ1 tyrosine phosphorylation and an increase in serine phosphorylation induced by ligation of the TCR/CD3 complex in Jurkat T cells (141). The decrease in the extent of IP3 production mediated by PMA (225) might be due to regulation of the activity of PLC-γ1 by phosphorylation on serine or threonine, but it also is possible that Shc regulates PLC-γ1 activity. The feedback inhibition of PLC-γ1 by activation of PKC is highly suggested to result in the rapid and transient $[Ca^{2+}]_i$ flux followed stimulation of FcγRIIA. Finally, it is interesting to speculate that the activation of PKC, inhibition of $[Ca^{2+}]_i$ flux, and Shc phosphorylation we observe following treatment with PMA may have a parallel in the normal regulation of $[Ca^{2+}]_i$ flux, since the diacylglycerol and IP3 formed by cleavage of PIP2 would activate PKC and might similarly inhibit PLC-γ1 and Shc

phosphorylation.

Materials and Methods

Immune complex internalization assay

Transfected cells in suspension were incubated with mAb IV.3 Fab (1 μg/ml, 30 min, 4^0C), washed, and then incubated with goat $F(ab')_2$ anti-mouse IgG (2 μg/ml, 20 min, 4^0C) to crosslink the transfected FcγRIIA. The cells were then shifted to 37^0C for the indicated intervals and washed at 4^0C prior to labeling with FITC-conjugated rabbit $F(ab')_2$ anti-goat IgG (30 μg/ml, 30 min, 4^0C) as detailed previously (149).

$[Ca^{2+}]_i$ flux assay

Cells in suspension (10^7/ml) were incubated with 5 μg/ml of Indo-1-AM (Molecular Probes, Inc., Eugene, OR) for 15 min at 37^0C, washed and resuspended in Ca^{2+} and Mg^{2+}-free physiological saline containing 5 mM KCl and 5 mM glucose, incubated with mAb IV.3 Fab (1 μg/ml, 37^0C, 5 min), washed, and resuspended in saline buffer containing 2 mM $MgCl_2$ and 1.2 mM $CaCl_2$ for 5 min. The stirred cell suspension was then transferred to a fluorimeter to determine the resting fluorescence emission ratio (405 nm/490 nm) during excitation at 355 nm. After 60 seconds, goat $F(ab')_2$ anti-mouse IgG (35 μg/ml final concentration) (Jackson ImmunoResearch, Inc.) was added. The maximal emission ratio was determined by lysing cells in 1% Triton X-100, and the minimal ratio by adding EDTA (40 mM final concentration). Indo-1 fluorescence emission ratios were converted to $[Ca^{2+}]_i$ as described previously (192).

Inhibitor studies

Cells coated with IV.3 Fab were preincubated with PMA (50-200 ng/ml, 15 min, Sigma), calphostin C (0.5-8 μM, 30 min, Sigma), H8 (1, 25 μM, 2 hr, Sigma), or cyclosporin A (0.5-4 μg/ml, 2 hr, Sigma) at 37^{0}C before crosslinking with goat $F(ab')_2$ anti-mouse IgG. In all pharmacologic studies, the agent used for pretreatment was continuously present during stimulation.

IV. General Discussion

It is still not clear how crosslinking or aggregation results in physiological signaling. The minimal effective trigger element appears to require a conserved motif first recognized by Reth (11). This peptide sequence contains two Y-X-X-L/I residues separated by 10 or 11 residues (Fig. 1A). Crosslinking of receptors bearing this element initiates an activation cascade for which protein tyrosine kinase activity appears to be essential since PTK inhibitors block both early events such as calcium mobilization and the later stimulus of cytokine release and cellular proliferation in T and B cell activation (241,242).

Much investigation has focused on identification of the PTKs involved in this proximal signaling pathway. Protein tyrosine kinases have been reported to associate with receptors that lack an intracellular catalytic domain. Lck, a Src family member, is associated with the coreceptors CD4 and CD8 through cysteine residues in N-terminal domain of Lck and the cytoplasmic domains of CD4 and CD8 (243). In a CD4-deficient T cell hybridoma, only forms of CD4 that couple with Lck can restore antigen-induced activation (244). A critical function of Lck in TCR signal transduction is suggested by genetic studies. A mutant leukemic cell, lacking functional Lck, did not flux intracellular calcium and/or activate the TCR-mediated PTK pathway (108). Overexpression of Fyn in transgenic mice leads to an antigen-hyperresponsive phenotype in the resulting T cells, while overexpression of the inactive kinase form blocks TCR-mediated activation (245). Thymic T cells isolated from knock-out mice lacking Fyn kinase activity show a profound defect in

proliferation in response to treatment with a combination of phorbol ester plus either anti-CD3 antibody or concanavalin A (106,246). The B cell antigen receptor complex is a hetero-oligomeric structure composed of the antigen recognition element, membrane immunoglobulin (mIg), and transducer elements. Proteins that transduce the signal include the disulfide-linked heterodimers of Ig-α and Ig-β/γ subunits, products of the mb-1 (α) and B-29 (β/γ) genes (247,248). The antigen receptor homology motif (ARH1) of Ig-α and Ig-β were expressed as fusion proteins with glutathione-S-transferase (GST) and these proteins were coupled to glutathione Sepharose beads. These constructs were used as probes to identify molecules that can bind the receptor complex. The ARH1 of Ig-α chain bound to the Src family kinases Lyn and Fyn, phosphatidylinositol-3 kinase (PI-3 kinase) though the ARH1 of Ig-β bound PI-3 kinase (98).

Recently, a new family of PTK has been shown to be involved in B and T cell activation. The $p72^{syk}$ was found to associate with membrane Ig and to be phosphorylated upon crosslinking with anti-IgM antibodies (208). ZAP-70, a second member of the Syk family, has been shown to associate with the ζ chain of TCR/CD3 following crosslinking of receptor (196). Engagement of TCR/CD3 also results in association of ZAP-70 to the ϵ chain of CD3 (209). In a recent study, the tandem SH2 domains of ZAP-70, expressed as a glutathione S-transferase (GST) fusion protein, bind to tyrosine-phosphorylated ζ and ϵ chains of TCR/CD3 from activated Jurkat T cell lysates. Neither N- nor C-terminal SH2 domains of ZAP-70 exhibit such binding activity (249).

More recently, Kolanus et al. (250) addressed the possibility that nonreceptor kinases might directly activate cells by membrane clustering events. To test this they created artificial receptor kinase chimeras by replacing the intracellular domains of nonkinase receptors with the complete Src or Syk family kinases sequences and examined the sequential events of aggregation of these chimera receptors by extracellular crosslinking in T cells. The chimeras containing Src family kinases Lck and Fyn did not lead to cellular activation (calcium mobilization and cytolytic effect), whereas Syk or ZAP-70 chimeras triggered calcium mobilization with a slight delay. Aggregation of the Syk chimera alone, or coaggregation of ZAP-70 and Fyn chimeras, activated cytolytic effector function. Only the pattern of tyrosine phosphorylation induced by crosslinking of the Syk chimera was similar to the pattern induced by aggregation of T cell receptor.

Protein-tyrosine kinases have also been reported to associate with Fc receptors and to be involved in the signal transduction upon crosslinking of receptor. $p72^{syk}$ participates in signaling by FcγRI and FcγRII in HL60 cells (121). $p56^{lyn}$ and $pp60^{c\text{-}src}$ were activated after FcϵRI cross-linking and $p56^{lyn}$ was found to coimmunoprecipitate with FcϵRI in RBL-2H3 cells. In the mast cell line PT-18, $p62^{c\text{-}yes}$ was activated upon FcϵRI engagement and coimmunoprecipitated with the receptor (118). Src-like protein-tyrosine kinase Fgr was shown to associate and to be activated after crosslinking of FcγRII in neutrophils (117).

Although most research of signal transduction to date has focused on the activation of PTKs, evidence is beginning to accumulate regarding the regulation of

protein tyrosine phosphatases (PTPs). The important role of CD45 in TCR signaling has been discussed (see background). Recently, Desai et al. (251) showed that a chimeric protein, consisting of the cytoplasmic domain of CD45 and the extracellular and transmembrane domains with those of the epidermal growth factor receptor (EGFR), was able to restore TCR signaling in a CD45-deficient cell. Thus, the cytoplasmic domain of CD45 is sufficient for TCR signaling. Moreover, engagement of EGFR-CD45 chimera by EGF resulted in the loss of TCR signaling. These results indicate that CD45 function is continuously required for TCR signaling. In $CD45^{-exon6-/-}$ mice, B cells and most T cells did not express CD45. T cell development was blocked at the transitional stage from immature $CD4^+CD8^+$ to mature $CD4^+$ or $CD8^+$ cells, and the number of peripheral T cells was significantly reduced. B cell proliferation induced by surface IgM receptor engagement was completely abrogated, although development of B cells appeared normal (252).

In addition, cytoplasmic PTPs also participate in receptor signaling. EGF stimulated not only tyrosine phosphorylation but also PTP activity. The PTP activity was localized in the cytosol and selective toward ErbB2 and EGFR, but not phospholipase C-γ1 and Ras GTPase-activating protein (253). There are two classes of mammalian PTPs containing SH2 domains. One class is specifically expressed in hematopoietic cells (PTP1C, SH-PTP1, HCP, and SHP) (254,255), whereas the other class (PTP1D, SH-PTP2, SH-PTP3, and Syp) (256,257) appears to be ubiquitous. Syp physically associates the activated EGFR and PDGFR *in vitro* and *in vivo* through SH2 domains and is tyrosine phosphorylated in EGF- or PDGF-stimulated cells or v-

src-transformed cells (256-258). HCP or PTP1C transiently associates with ligand-activated c-Kit but not c-Fms through the SH2 domains and is tyrosine phosphorylated in colony-stimulating factor 1- or stem cell factor-stimulated cells (259). An unidentified PTP coprecipitated with hepatocyte growth factor/scatter factor (HGF/SF) receptor, and the PTP activity increased after HGF/SF stimulation (260).

In our studies, crosslinking of the Y252F mutant resulted in somewhat slower tyrosine phosphorylation and markedly slower dephosphorylation of most of the cellular substrates, suggesting that the Tyr^{252} is important for protein tyrosine phosphatase binding and/or activation. In addition, phenylarsine oxide (PAO) and pervanadate, protein tyrosine phosphatase inhibitors, inhibited function and tyrosine phosphorylation induced by receptor crosslinking. Thus we believe that receptor, protein tyrosine phosphatases (PTPs), protein tyrosine kinases (PTKs), and other effectors are associated to initiate signal transduction, and suggest that the activation of PTPs may be required to activate PTKs. Protein tyrosine phosphatases and protein tyrosine kinases associated with the receptor complex have been tested by immunoprecipitation of FcγRIIA or absorption of GST-FcγRIIA cytoplasmic domain fusion protein followed *in vitro* kinase assays and immunoblotting with anti-phosphatase sera and the anti-phosphotyrosine mAb 4G10. I have also attempted to identify the receptor associated PTPs and PTKs by immunoblotting with specific antibodies following immunoprecipitation of FcγRIIA. However, no convincing result has been obtained from these experiments.

V. Bibliography

1. Teillaud, J. L., C. Mathiot, S. Ameigorena, S. Brunati, J. Moncuit, and W. H. Fridman. 1987. Regulation of hybridoma B cell proliferation by immunoglobulin binding factor (IBF). In Cancer Detection and Prevention. Anonymous, editor. Liss, New York.

2. Phillips, N. E. and D. C. Parker. 1985. Subclass specificity of Fc gamma receptor-mediated inhibition of mouse B cell activation. *J. Immunol.* 134:2835.

3. Stuart, S. G., N. E. Simister, S. B. Clarkson, B. M. Kacinski, M. Shapiro, and I. Mellman. 1989. Human IgG Fc receptor (hFcRII; CD32) exists as multiple isoforms in macrophages, lymphocytes and IgG-transporting placental epithelium. *EMBO J.* 8:3657.

4. Boros, P. , J. Chen, C. Bona, and J. C. Unkeless. 1990. Autoimmune mice make anti-Fc gamma receptor Ig. *J. Exp. Med.* 171:1581.

5. Sipos, A. , C. Csortos, S. Sipka, P. Gergely, I. Sonkoly, and G. Szegedi. 1988. The antigen/receptor specificity of antigranulocyte antibodies in patients with SLE. *Immunol. Lett.* 19:329.

6. Ravetch, J. V. and J. -P. Kinet. 1991. Fc receptors. *Annu. Rev. Immunol.* 9:457.

7. Unkeless, J. C., P. Boros, and M. Fein. 1992. Structure, signalling and function of Fc gamma receptors. In Inflammation: Basic Principles and Clinical Correlates. J. I. Gallin and I. M. Goldstein, editors. Raven Press, Ltd., New York. 497-510.

8. Metzger, H. and J. P. Kinet. 1988. How antibodies work: focus on Fc receptors. *FASEB. J.* 2:3.

9. Williams, A. F. and A. N. Barclay. 1988. The immunoglobulin superfamily--domains for cell surface recognition. *Annu. Rev. Immunol.* 6:381.

10. Selvaraj, P. , W. F. Rosse, R. Silber, and T. A. Springer. 1988. The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal haemoglobinuria. *Nature* 333:565.

11. Reth, M . 1989. Antigen receptor tail clue. *Nature* 338:383.

12. Keegan, A. D. and W. E. Paul. 1992. Multichain immune recognition receptors: Similarities in structure and signaling pathways. *Immunol. Today* 13:63.

13. Irving, B. A., A. C. Chan, and A. Weiss. 1993. Functional characterization of a

signal transducing motif present in the T cell antigen receptor zeta chain. *J Exp. Med.* 177:1093.

14. Ernst, L. K., J. G. van de Winkel, I. M. Chiu, and C. L. Anderson. 1992. Three genes for the human high affinity Fc receptor for IgG (Fc gamma RI) encode four distinct transcription products. *J. Biol. Chem.* 267:15692.

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

16. Hulett, M. D., N. Osman, I. F. C. McKenzie, and P. M. Hogarth. 1991. Chimeric Fc receptors identify functional domains of the murine high affinity receptor for IgG. *J. Immunol.* 147:1863.

17. Sears, D. W., N. Osman, B. Tate, I. F. C. McKenzie, and P. M. Hogarth. 1990. Molecular cloning and expression of the mouse high affinity Fc receptor for IgG. *J. Immunol.* 144:371.

18. Ceuppens, J. L., M. L. Baroja, F. Van Vaeck, and C. L. Anderson. 1988. Defect in the membrane expression of high affinity 72-kD Fc gamma receptors on phagocytic cells in four healthy subjects. *J. Clin. Invest.* 82:571.

19. Looney, R. J., G. N. Abraham, and C. L. Anderson. 1986. Human monocytes and U937 cells bear two distinct Fc receptors for IgG. *J. Immunol.* 136:1641.

20. Antoun, G. R., B. M. Longenecker, and T. F. Zipf. 1989. Comparison of the 40 kDa hematopoietic cell antigens bound by monoclonal antibodies IV.3, 41H.16 and KB61. *Mol. Immunol.* 26:333.

21. Stuart, S. G., M. L. Trounstine, D. J. Vaux, T. Koch, C. L. Martens, I. Mellman, and K. W. Moore. 1987. Isolation and expression of cDNA clones encoding a human receptor for IgG (Fc gamma RII). *J. Exp. Med.* 166:1668.

22. Brooks, D. G., W. Q. Qiu, A. D. Luster, and J. V. Ravetch. 1989. Structure and expression of human IgG FcRII(CD32). Functional heterogeneity is encoded by the alternatively spliced products of multiple genes. *J. Exp. Med.* 170:1369.

23. Ravetch, J. V., A. D. Luster, R. Weinshank, J. Kochan, A. Pavlovec, D. A. Portnoy, J. Hulmes, Y. C. Pan, and J. C. Unkeless. 1986. Structural heterogeneity and functional domains of murine immunoglobulin G Fc receptors. *Science* 234:718.

24. Warmerdam, P. A. M., J. G. van de Winkel, E. J. Gosselin, and P. J. A. Capel. 1990. Molecular basis for a polymorphism of human Fcgamma receptor II (CD32). *J. Exp. Med.* 172:19.

25. Clark, M. R., S. B. Clarkson, P. A. Ory, N. Stollman, and I. M. Goldstein. 1989. Molecular basis for a polymorphism involving Fc receptor II on human monocytes. *J. Immunol.* 143:1731.

26. Looney, R. J., C. L. Anderson, D. H. Ryan, and S. I. Rosenfeld. 1988. Structural polymorphism of the human platelet Fc gamma receptor. *J. Immunol.* 141:2680.

27. Parren, P. W., P. A. Warmerdam, L. C. Boeije, J. Arts, N. A. Westerdaal, A. Vlug, P. J. Capel, L. A. Aarden, and J. G. van de Winkel. 1992. On the interaction of IgG subclasses with the low affinity Fc gamma RIIa (CD32) on human monocytes, neutrophils, and platelets. Analysis of a functional polymorphism to human IgG2. *J. Clin. Invest.* 90:1537.

28. Scallon, B. J., E. Scigliano, V. H. Freedman, M. C. Miedel, Y. -C. E. Pan, J. C. Unkeless, and J. P. Kochan. 1989. A human immunoglobulin G receptor exists in both polypeptide-anchored and phosphatidylinositol-glycan-anchored forms. *Proc. Natl. Acad. Sci. USA* 86:5079.

29. Ueda, E. , T. Kinoshita, J. Nojima, K. Inoue, and T. Kitani. 1989. Different membrane anchors of FCgammaRIII (CD16) on K/NK-lymphocytes and neutrophils: Protein-vs lipid-anchor. *J. Immunol.* 143:1274.

30. Metzger, H . 1992. The receptor with high affinity for IgE. *Immunol. Rev.* 125:37.

31. Ra, C. , M. -H. E. Jouvin, U. Blank, and J. -P. Kinet. 1989. A macrophage Fcgamma receptor and the mast cell receptor for IgE share an identical subunit. *Nature* 341:752.

32. Letourneur, O. , I. C. Kennedy, A. T. Brini, J. R. Ortaldo, J. J. O'Shea, and J. P. Kinet. 1991. Characterization of the family of dimers associated with Fc receptors (Fc epsilon RI and Fc gamma RIII). *J. Immunol.* 147:2652.

33. Selvaraj, P. , O. Carpen, M. L. Hibbs, and T. A. Springer. 1989. Natural killer cell and granulocyte Fcgamma receptor III (CD16) differ in membrane anchor and signal transduction. *J. Immunol.* 143:3283.

34. Roberts, W. L., J. J. Myher, A. Kuksis, M. G. Low, and T. L. Rosenberry. 1988. Lipid analysis of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase. Palmitoylation of inositol results in resistance to phosphatidylinositol-specific phospholipase C. *J. Biol. Chem.* 263:18766.

35. Edberg, J. C., J. E. Salmon, M. Whitlow, and R. P. Kimberly. 1991. Preferential expression of human Fc gamma RIIIPMN (CD16) in paroxysmal nocturnal hemoglobinuria. Discordant expression of glycosyl phosphatidylinositol-linked proteins. *J Clin. Invest.* 87:58.

36. Lalezari, P. , M. Khorshidi, and M. Petrosova. 1986. Autoimmune neutropenia of infancy. *J. Pediatr.* 109:764.

37. Edberg, J. C., P. B. Redecha, J. E. Salmon, and R. P. Kimberly. 1989. Human FcgammaRIII (CD16). Isoforms with distinct allelic expression, extracellular domains, and membrane linkages on polymorphonuclear and natural killer cells. *J. Immunol.* 143:1642.

38. Berken, A. and B. Benacerraf. 1968. Sedimentation properties of antibody cytophilic for macrophages.. *J. Immunol.* 100:1219.

39. Walker, W. S. 1976. Separate Fc-receptors for immunoglobulins IgG2a and IgG2b on an established cell line of mouse macrophages.. *J. Immunol.* 116:911.

40. Heusser, C. H., C. L. Anderson, and H. M. Grey. 1977. Receptors for IfG. Subclass specificity of receptors on different mouse cell types and the definition of two distinct receptors on a macrophage cell line.. *J. Exp. Med.* 145:1316.

41. Unkeless, J. C. 1977. The presence of two Fc receptors on mouse macrophages: evidence from a variant cell line and differential trypsin sensitivity. *J. Exp. Med.* 145:931.

42. Diamond, B. , B. R. Bloom, and M. D. Scharff. 1978. The Fc receptors of primary and cultured phagocytic cells studied with homogenous antibodies.. *J. Immunol.* 121:1329.

43. Unkeless, J. C. and H. N. Eisen. 1975. Binding of monomeric immunoglobulins to Fc receptors of mouse macrophages. *J. Exp. Med.* 142:1520.

44. Howard, J. G. and B. Benacerraf. 1966. Properties of macrophage receptors for cytophilic antibodies.. *Br. J. Exp. Pathol.* 47:193.

45. Lesile, R. G. Q. and S. Cohen. 1974. Cytophilic activity of IgG2 from the sera of unimmunized guinea pigs.. *Immunology* 27:577.

46. Arend, W. P. and M. Mannik. 1972. In vitro adherence of soluable immune complexes to macrophages.. *J. Exp. Med.* 136:514.

47. Unkeless, J. C. 1979. Characterization of a monoclonal antibody directed against

mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med.* 150:580.

48. Poglitsch, C. L. and N. L. Thompson. 1990. Interaction of antibodies with Fc receptors in substrate-supported planar membranes measured by total internal reflection fluorescence microscopy. *Biochemistry* 29:248.

49. Mellman, I. S. and J. C. Unkeless. 1980. Purificaton of a functional mouse Fc receptor through the use of a monoclonal antibody. *J. Exp. Med.* 152:1048.

50. Qu, Z. , J. Odin, J. D. Glass, and J. C. Unkeless. 1988. Expression and characterization of a truncated murine Fcgamma receptor. *J. Exp. Med.* 167:1195.

51. Hogarth, P. M., M. L. Hibbs, L. Bonadonna, B. M. Scott, E. Witort, G. A. Pietersz, and I. F. McKenzie. 1987. The mouse Fc receptor for IgG (Ly-17): molecular cloning and specificity. *Immunogenetics.* 26:161.

52. Weinshank, R. L., A. D. Luster, and J. V. Ravetch. 1988. Function and regulation of a murine macrophage-specific IgG Fc receptor, Fc gamma R-alpha. *J. Exp. Med.* 167:1909.

53. Kulczycki, A. , Jr., V. Krause, C. C. Killion, and J. P. Atkinson. 1980. Purification of Fc gamma receptor from rabbit alveolar macrophages that retains ligand-binding activity.. *J. Immunol.* 124:2772.

54. Schneck, J. , O. M. Rosen, B. Diamond, and B. R. Bloom. 1981. Modulation of Fc-receptor expression and Fc-mediated phagocytosis in variants of a macrophage-like cell line. *J. Immunol.* 126:745.

55. Lewis, V. A., T. Koch, H. Plutner, and I. Mellman. 1986. A complementary DNA clone for a macrophage-lymphocyte Fc receptor. *Nature* 324:372.

56. Hibbs, M. L., B. J. Classon, I. D. Walker, I. F. McKenzie, and P. M. Hogarth. 1988. The structure of the murine Fc receptor for IgG. Assignment of intrachain disulfide bonds, identification of N-linked glycosylation sites, and evidence for a fourth form of Fc receptor. *J. Immunol.* 140:544.

57. Hogarth, P. M., N. Osman, D. W. Sears, M. Vovos, C. E. Witort, J. Even, and I. F. McKenzie. 1989. Molecular analysis of FcR of T cells and other cell types. *Transplant. Proc.* 21:44.

58. Bonnerot, C. , M. Daeron, N. Varin, S. Amigorena, P. M. Hogarth, J. Even, and W. H. Fridman. 1988. Methylation in the 5' region of the murine beta Fc gamma R gene regulates the expression of Fc gamma receptor II. *J. Immunol.* 141:1026.

59. Perussia, B. , M. M. Tutt, W. Q. Qiu, W. A. Kuziel, P. W. Tucker, G. Trinchieri, M. Bennett, J. V. Ravetch, and V. Kumar. 1989. Murine natural killer cells express functional Fcgamma receptor II encoded by the FcgammaRα gene. *J. Exp. Med.* 170:73.

60. Schreiber, R. E., A. Buku, and J. C. Unkeless. 1990. Expression of murine Fc receptors for IgG. *J. Immunol.* 144:4735.

61. Bonnerot, C. , S. Amigorena, W. H. Fridman, J. Even, and M. Daëron. 1990. Unmethylation of specific sites in the 5' region is critical for the expression of murine αFcgammaR gene. *J. Immunol.* 144:323.

62. Santiago, A. , J. Satriano, S. DeCandido, H. Holthofer, R. Schreiber, J. Unkeless, and D. Schlondorff. 1989. A specific Fc gamma receptor on cultured rat mesangial cells. *J. Immunol.* 143:2575.

63. Daeron, M. , C. Bonnerot, S. Latour, M. Benhamou, and W. H. Fridman. 1990. Identification of Fc gamma RIIa, a product of the murine alpha Fc gamma R gene. *Eur. J. Immunol.* 20:897.

64. Guyre, P. M., P. M. Morganelli, and R. Miller. 1983. Recombinant immune interferon increases immunoglobulin G Fc receptors on cultured human mononuclear phagocytes. *J. Clin. Invest.* 72:393.

65. Perussia, B. , E. T. Dayton, R. Lazarus, V. Fanning, and G. Trinchieri. 1983. Immune interferon induces the receptor for monomeric IgG1 on human monocytic and myeloid cells. *J. Exp. Med.* 158:1092.

66. Akiyama, Y. , M. D. Lubeck, Z. Steplewski, and H. Koprowski. 1984. Induction of mouse IgG2a- and IgG3-dependent cellular cytotoxicity in human monocytic cells (U937) by immune interferon. *Cancer Research* 44:5127.

67. Maluish, A. E., W. J. Urba, D. L. Longo, W. R. Overton, D. Coggin, E. R. Crisp, R. Williams, S. A. Sherwin, K. Gordon, and R. G. Steis. 1988. The determination of an immunologically active dose of interferon-gamma in patients with melanoma. *J. Clin. Oncol.* 6:434.

68. Guyre, P. M., A. S. Campbell, W. D. Kniffin, and M. W. Fanger. 1990. Monocytes and polymorphonuclear neutrophils of patients with streptococcal pharyngitis express increased numbers of type I IgG Fc receptors. *J. Clin. Invest.* 86:1892.

69. Shen, L. , C. R. Maliszewski, W. F. Rigby, and M. W. Fanger. 1986. IgA-mediated effector function of HL-60 cells following treatment with calcitriol.

Mol. Immunol. 23:611.

70. Fries, L. F., C. M. Brickman, and M. M. Frank. 1983. Monocyte receptors for the Fc portion of IgG increase in number in autoimmune hemolytic anemia and other hemolytic states and are decreased by glucocorticoid therapy.. *J. Immunol.* 131:1240.

71. Girard, M. T., S. Hjaltadottir, T. A. N. Fejes, and P. M. Guyre. 1987. Glucocorticoids enhance the gamma-interferon augmentation of human monocyte immunoglobulin G Fc receptor expression. *J. Immunol.* 138:3235.

72. Schreiber, A. D., P. Chien, A. Tomaski, and D. B. Cines. 1987. Effect of danazol in immune thrombocytopenic purpura. *N. Engl. J. Med.* 316:503.

73. Crabtree, G. R., A. Munck, and K. A. Smith. 1979. Glucocorticoids inhibit expression of Fc receptors on the human granulocytic cell line HL-60.. *Nature* 279:338.

74. Petroni, K. C., L. Shen, and P. M. Guyre. 1988. Modulation of human polymorphonuclear leukocyte IgG Fc receptors and Fc receptor-mediated functions by IFN-gamma and glucocorticoids. *J. Immunol.* 140:3467.

75. Fleit, H. B., S. D. Wright, C. J. Durie, J. E. Valinsky, and J. C. Unkeless. 1984. Ontogeny of Fc receptors and complement receptor (CR3) during human myeloid differentiation. *J. Clin. Invest.* 73:516.

76. Passlick, B. , D. Flieger, and H. W. Ziegler-Heitbrock. 1989. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood* 74:2527.

77. Welch, G. R., H. L. Wong, and S. M. Wahl. 1990. Selective induction of FcgammaRIII on human monocytes by transforming growth factor-β. *J. Immunol.* 144:3444.

78. Nambu, M. , M. Morita, H. Watanabe, Y. Uenoyama, K. -M. Kim, M. Tanaka, Y. Iwai, H. Kimata, M. Mayumi, and H. Mikawa. 1989. Regulation of Fcgamma receptor expression and phagocytosis of a human monoblast cell line U937: Participation of cAMP and protein kinase C in the effects of IFN-gamma and phorbol ester. *J. Immunol.* 143:4158.

79. Arulanandam, A. R. N., S. Koyasu, and E. L. Reinherz. 1991. T cell receptor-independent CD2 signal transduction in FcR^+ cells. *J. Exp. Med.* 173:859.

80. Laszlo, G. and H. B. Dickler. 1988. IL-4 induces loss of B lymphocyte Fc

gamma R II ligand binding capacity. *J. Immunol.* 141:3416.

81. Phillips, N. E., K. A. Gravel, K. Tumas, and D. C. Parker. 1989. IL-4 (B cell stimulatory factor 1) overcomes Fc gamma receptor-mediated inhibition of mouse B lymphocyte proliferation without affecting inhibition of c-myc mRNA induction. *J. Immunol.* 141:4243.

82. Avalos, B. R., J. C. Gasson, C. Hedvat, S. G. Quan, G. C. Baldwin, R. H. Weisbart, R. E. Williams, D. W. Golde, and J. F. DiPersio. 1990. Human granulocyte colony-stimulating factor: biologic activities and receptor characterization on hematopoietic cells and small cell lung cancer cell lines. *Blood* 75:851.

83. Roberts, P. J., S. Devereux, G. R. Pilkington, and D. C. Linch. 1990. Fc gamma RII-mediated superoxide production by phagocytes is augmented by GM-CSF without a change in Fc gamma RII expression. *J. Leukoc. Biol.* 48:247.

84. Atkinson, Y. H., W. A. Marasco, A. F. Lopez, and M. A. Vadas. 1988. Recombinant human tumor necrosis factor-alpha. Regulation of N-formylmethionylleucylphenylalanine receptor affinity and function on human neutrophils. *J. Clin. Invest.* 81:759.

85. Berkow, R. L., D. Wang, J. W. Larrick, R. W. Dodson, and T. H. Howard. 1987. Enhancement of neutrophil superoxide production by preincubation with recombinant human tumor necrosis factor. *J. Immunol.* 139:3783.

86. Graziano, R. F., R. J. Looney, L. Shen, and M. W. Fanger. 1989. Fc gamma R-mediated killing by eosinophils. *J. Immunol.* 142:230.

87. Anegon, I. , M. C. Cuturi, G. Trinchieri, and B. Perussia. 1988. Interaction of Fc receptor (CD16) ligands induces transcription of interleukin 2 receptor (CD25) and lymphokine genes and expression of their products in human natural killer cells. *J. Exp. Med.* 167:452.

88. Cassatella, M. A., I. Anegon, M. C. Cuturi, P. Griskey, G. Trinchieri, and B. Perussia. 1989. Fc gamma R(CD16) interaction with ligand induces Ca2+ mobilization and phosphoinositide turnover in human natural killer cells. Role of Ca2+ in Fc gamma R(CD16)-induced transcription and expression of lymphokine genes. *J. Exp. Med.* 169:549.

89. Debets, J. M., J. G. van de Winkel, J. L. Ceuppens, I. E. Dieteren, and W. A. Buurman. 1990. Cross-linking of both Fc gamma RI and Fc gamma RII induces secretion of tumor necrosis factor by human monocytes, requiring high affinity Fc-Fc gamma R interactions. Functional activation of Fc gamma RII by treatment with proteases or neuraminidase. *J. Immunol.* 144:1304.

90. Debets, J. M., C. J. van der Linden, I. E. Dieteren, J. F. Leeuwenberg, and W. A. Buurman. 1988. Fc-receptor cross-linking induces rapid secretion of tumor necrosis factor (cachectin) by human peripheral blood monocytes. *J. Immunol.* 141:1197.

91. Ernst, L. K., A. -M. Duchemin, and C. L. Anderson. 1993. Association of the high-affinity receptor for IgG (FcgammaRI) with the gamma subunit of the IgE receptor. *Proc. Natl. Acad. Sci. USA* 90:6023.

92. Hibbs, M. L., P. Selvaraj, O. Carpen, T. A. Springer, H. Kuster, M. -H. E. Jouvin, and J. -P. Kinet. 1989. Mechanisms for regulating expression of membrane isoforms of FcgammaRIII (CD16). *Science* 246:1608.

93. Schoneich, J. T., V. L. Wilkinson, H. Kado Fong, D. H. Presky, and J. P. Kochan. 1992. Association of the human Fc epsilon RI gamma subunit with novel cell surface polypeptides. *J. Immunol.* 148:2181.

94. Kurosaki, T. , I. Gander, and J. V. Ravetch. 1991. A subunit common to an IgG Fc receptor and the T-cell receptor mediates assembly through different interactions. *Proc. Natl. Acad. Sci. USA* 88:3837.

95. Kurosaki, T. and J. V. Ravetch. 1989. A single amino acid in the glycosyl phosphatidylinositol attachment domain determines the membrane topology of FcgammaRIII. *Nature* 342:805.

96. Kinet, J. P. 1992. The gamma-zeta dimers of Fc receptors as connectors to signal transduction. *Curr. Opin. Immunol.* 4:43.

97. Samelson, L. E. and R. D. Klausner. 1992. Tyrosine kinases and tyrosine-based activation motifs. Current research on activation via the T cell antigen receptor. *J. Biol. Chem.* 267:24913.

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

99. Huang, M. M., Z. Indik, L. F. Brass, J. A. Hoxie, A. D. Schreiber, and J. S. Brugge. 1992. Activation of Fc gamma RII induces tyrosine phosphorylation of multiple proteins including Fc gamma RII. *J. Biol. Chem.* 267:5467.

100. June, C. H., M. C. Fletcher, J. A. Ledbetter, and L. E. Samelson. 1990. Increases in tyrosine phosphorylation are detectable before phospholipase C activation

after T cell receptor stimulation. *J. Immunol.* 144:1591.

101. Mustelin, T. , K. M. Coggeshall, N. Isakov, and A. Altman. 1990. T cell antigen receptor-mediated activation of phospholipase C requires tyrosine phosphorylation. *Science* 247:1584.

102. Azzoni, L. , M. Kamoun, T. W. Salcedo, P. Kanakaraj, and B. Perussia. 1993. Stimulation of Fc gamma RIIIA results in phospholipase C-gamma1 tyrosine phosphorylation and p56(lck) activation. *J. Exp. Med.* 176:1745.

103. Benhamou, M. , V. Stephan, K. C. Robbins, and R. P. Siraganian. 1992. High-affinity IgE receptor-mediated stimulation of rat basophilic leukemia (RBL-2H3) cells induces early and late protein-tyrosine phosphorylations. *J. Biol. Chem.* 267:7310.

104. Alber, G. , U. M. Kent, and H. Metzger. 1992. Functional comparison of Fc epsilon RI, Fc gamma RII, and Fc gamma RIII in mast cells. *J. Immunol.* 149:2428.

105. Eiseman, E. and J. B. Bolen. 1990. src-related tyrosine protein kinases as signaling components in hematopoietic cells [published erratum appears in Cancer Cells 1990 Dec;2(12):405]. *Cancer Cells* 2:303.

106. Appleby, M. W., J. A. Gross, M. P. Cooke, S. D. Levin, X. Qian, and R. M. Perlmutter. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of p59fyn. *Cell* 70:751.

107. Veillette, A. , M. A. Bookman, E. M. Horak, and J. B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. *Cell* 55:301.

108. Straus, D. B. and A. Weiss. 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell* 70:585.

109. Veillette, A. , M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen. 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56lck. *Nature* 338:257.

110. Veillette, A. , J. B. Bolen, and M. A. Bookman. 1989. Alterations in tyrosine protein phosphorylation induced by antibody-mediated cross-linking of the CD4 receptor of T lymphocytes. *Mol. Cell Biol.* 9:4441.

111. Chan, A. C., M. Iwashima, C. W. Turck, and A. Weiss. 1992. ZAP-70: a

70-kD protein tyrosine kinase that associates with the TCR zeta chain. *Cell* 71:649.

112. Gold, M. R., L. Matsuuchi, R. B. Kelly, and A. L. DeFranco. 1991. Tyrosine phosphorylation of components of the B-cell antigen receptors following receptor crosslinking. *Proc. Natl. Acad. Sci. USA* 88:3436.

113. Dymecki, S. M., J. E. Niederhuber, and S. V. Desiderio. 1990. Specific expression of a tyrosine kinase gene, blk, in B lymphoid cells. *Science* 19; 247:332.

114. Yamanashi, Y. , T. Kakiuchi, J. Mizuguchi, T. Yamamoto, and K. Toyoshima. 1991. Association of B cell antigen receptor with protein tyrosine kinase Lyn. *Science* 251:192.

115. Burkhardt, A. L., M. Brunswick, J. B. Bolen, and J. J. Mond. 1991. Anti-immunoglobulin stimulation of B lymphocytes activates src-related protein-tyrosine kinases. *Proc. Natl. Acad. Sci. USA* 88:7410.

116. Salcedo, T. W., T. Kurosaki, P. Kanakaraj, J. V. Ravetch, and B. Perussia. 1993. Physical and functional association of $p56^{lck}$ with FcgammaRIIIA (CD16) in natural killer cells. *J. Exp. Med.* 177:1475.

117. Hamada, F. , M. Aoki, T. Akiyama, and K. Toyoshima. 1993. Association of immunoglobulin G Fc receptor II with Src-like protein-tyrosine kinase Fgr in neutrophils. *Proc. Natl. Acad. Sci. USA* 90:6305.

118. Eiseman, E. and J. B. Bolen. 1992. Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases. *Nature* 355:78.

119. Yamada, T. , T. Taniguchi, C. Yang, S. Yasue, H. Saito, and H. Yamamura. 1993. Association with B-cell-antigen receptor with protein-tyrosine kinase $p72^{syk}$ and activation by engagement of membrane IgM. *Eur. J. Biochem.* 213:455.

120. Hutchcroft, J. E., R. L. Geahlen, G. G. Deanin, and J. M. Oliver. 1992. Fc epsilon RI-mediated tyrosine phosphorylation and activation of the 72-kDa protein-tyrosine kinase, PTK72, in RBL-2H3 rat tumor mast cells. *Proc. Natl. Acad. Sci. USA* 89:9107.

121. Agarwal, A. , P. Salem, and K. C. Robbins. 1993. Involvement of $p72^{syk}$, a protein-tyrosine kinase, in Fcgamma receptor signaling. *J. Biol. Chem.* 268:15900.

122. Pingel, J. T. and M. L. Thomas. 1989. Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. *Cell* 58:1055.

123. Ledbetter, J. A., N. K. Tonks, E. H. Fischer, and E. A. Clark. 1988. CD45 regulates signal transduction and lymphocyte activation by specific association with receptor molecules on T or B cells. *Proc. Natl. Acad. Sci. USA* 85:8628.

124. Ostergaard, H. L. and I. S. Trowbridge. 1990. Coclustering CD45 with CD4 or CD8 alters the phosphorylation and kinase activity of p56lck. *J. Exp. Med.* 172:347.

125. Justement, L. B., K. S. Campbell, N. C. Chien, and J. C. Cambier. 1991. Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. *Science* 252:1839.

126. Hook, W. A., E. H. Berenstein, F. U. Zinsser, C. Fischler, and R. P. Siraganian. 1991. Monoclonal antibodies to the leukocyte common antigen (CD45) inhibit IgE-mediated histamine release from human basophils. *J. Immunol.* 147:2670.

127. Koch, C. A., D. Anderson, M. F. Moran, C. Ellis, and T. Pawson. 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science* 252:668.

128. Margolis, B . 1992. Proteins with SH2 domains: transducers in the tyrosine kinase signaling pathway. *Cell Growth Differ.* 3:73.

129. Fantl, W. J., J. A. Escobedo, G. A. Martin, C. W. Turck, M. del Rosario, F. McCormick, and L. T. Williams. 1992. Distinct phosphotyrosines on a growth factor receptor bind to specific molecules that mediate different signaling pathways. *Cell* 69:413.

130. Kashishian, A. , A. Kazlauskas, and J. A. Cooper. 1992. Phosphorylation sites in the PDGF receptor with different specificities for binding GAP and PI3 kinase in vivo. *EMBO J.* 11:1373.

131. Rotin, D. , B. Margolis, M. Mohammadi, R. J. Daly, G. Daum, N. Li, E. H. Fischer, W. H. Burgess, A. Ullrich, and J. Schlessinger. 1992. SH2 domains prevent tyrosine dephosphorylation of the EGF receptor: identification of Tyr992 as the high-affinity binding site for SH2 domains of phospholipase C gamma. *EMBO J.* 11:559.

132. Fukui, Y. , M. C. O'Brien, and H. Hanafusa. 1991. Deletions in the SH2 domain of p60v-src prevent association with the detergent-insoluble cellular matrix. *Mol. Cell Biol.* 11:1207.

133. Mayer, B. J., P. K. Jackson, R. A. Van Etten, and D. Baltimore. 1992. Point mutations in the abl SH2 domain coordinately impair phosphotyrosine binding in vitro

and transforming activity in vivo. *Mol. Cell Biol.* 12:609.

134. Matsuda, M. , C. T. Reichman, and H. Hanafusa. 1992. Biological and biochemical activity of v-Crk chimeras containing the SH2/SH3 regions of phosphatidylinositol-specific phospholipase C-gamma and Src. *J. Virol.* 66:115.

135. Ren, R. , B. J. Mayer, P. Cicchetti, and D. Baltimore. 1993. Identification of a ten-amino acid proline-rich SH3 binding site. *Science* 259:1157.

136. Duchesne, M. , F. Schweighoffer, F. Parker, F. Clerc, Y. Frobert, M. N. Thang, and B. Tocque. 1993. Identification of the SH3 Domain of GAP as an Essential Sequence for Ras-GAP-Mediated Signaling. *Science* 259:525.

137. Clark, S. G., M. J. Stern, and H. R. Horvitz. 1992. C. elegans cell-signalling gene sem-5 encodes a protein with SH2 and SH3 domains [see comments]. *Nature* 356:340.

138. Li, W. , G. G. Deanin, B. Margolis, J. Schlessinger, and J. M. Oliver. 1992. Fc epsilon R1-mediated tyrosine phosphorylation of multiple proteins, including phospholipase C gamma 1 and the receptor beta gamma 2 complex, in RBL-2H3 rat basophilic leukemia cells. *Mol. Cell Biol.* 12:3176.

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

140. Nishibe, S. , M. I. Wahl, S. M. Hernandez Sotomayor, N. K. Tonks, S. G. Rhee, and G. Carpenter. 1990. Increase of the catalytic activity of phospholipase C-gamma 1 by tyrosine phosphorylation. *Science* 250:1253.

141. Park, D. J., H. K. Min, and S. G. Rhee. 1992. Inhibition of CD3-linked phospholipase C by phorbol ester and by cAMP is associated with decreased phosphotyrosine and increased phosphoserine contents of PLC-gamma 1. *J. Biol. Chem.* 267:1496.

142. Paolini, R. and J. -P. Kinet. 1993. Cell surface control of the multiubiquination and deubiquination of high-affinity immunoglobulin E receptors. *EMBO J.* 12:779.

143. Kennerly, D. A. 1990. Phosphatidylcholine is a quantitatively more important source of increased 1,2-diacylglycerol than is phosphatidylinositol in mast cells. *J. Immunol.* 144:3912.

144. Lin, P. Y., G. A. Wiggan, and A. M. Gilfillan. 1991. Activation of phospholipase D in a rat mast (RBL 2H3) cell line. A possible unifying mechanism

for IgE-dependent degranulation and arachidonic acid metabolite release. *J. Immunol.* 146:1609.

145. Billah, M. M., S. Eckel, T. J. Mullmann, R. W. Egan, and M. I. Siegel. 1989. Phosphatidylcholine hydrolysis by phospholipase D determines phosphatidate and diglyceride levels in chemotactic peptide-stimulated human neutrophils. Involvement of phosphatidate phosphohydrolase in signal transduction. *J. Biol. Chem.* 264:17069.

146. Ting, A. T., L. M. Karnitz, R. A. Schoon, R. T. Abraham, and P. J. Leibson. 1992. Fc gamma receptor activation induces the tyrosine phosphorylation of both phospholipase C (PLC)-gamma 1 and PLC-gamma 2 in natural killer cells. *J. Exp. Med.* 176:1751.

147. Liao, F. , H. S. Shin, and S. G. Rhee. 1992. Tyrosine phosphorylation of phospholipase C-gamma 1 induced by cross-linking of the high-affinity or low-affinity Fc receptor for IgG in U937 cells. *Proc. Natl. Acad. Sci. USA* 89:3659.

148. Indik, Z. , C. Kelly, P. Chien, A. I. Levinson, and A. D. Schreiber. 1991. Human Fc gamma RII, in the absence of other Fc gamma receptors, mediates a phagocytic signal. *J. Clin. Invest.* 88:1766.

149. Odin, J. A., J. C. Edberg, C. J. Painter, R. P. Kimberly, and J. C. Unkeless. 1991. Regulation of phagocytosis and [Ca2+]i flux by distinct regions of an Fc receptor. *Science* 254:1785.

150. Tuijnman, W. B., P. J. A. Capel, and J. G. J. Van de Winkel. 1992. Human low-affinity IgG receptor FcgammaRIIa (CD32) introduced into mouse fibroblasts mediates phagocytosis of sensitized erythrocytes. *Blood* 79:1651.

151. Kolanus, W. , C. Romeo, and B. Seed. 1992. Lineage-independent activation of immune system effector function by myeloid Fc receptors. *EMBO J.* 11:4861.

152. Latour, S. , C. Bonnerot, W. H. Fridman, and M. Daeron. 1992. Induction of tumor necrosis factor-alpha production by mast cells via Fc gamma R. *J. Immunol.* 149:2155.

153. Miettinen, H. M., J. K. Rose, and I. Mellman. 1989. Fc receptor isoforms exhibit distinct abilities for coated pit localization as a result of cytoplasmic domain heterogeneity. *Cell* 58:317.

154. Joiner, K. A., S. A. Fuhrman, H. M. Miettinen, L. H. Kasper, and I. Mellman. 1990. Toxoplasma gondii: Fusion Competence of Parasitophorous Vacuoles in Fc Receptor-Transfected Fibroblasts. *Science* 249:641.

155. Amigorena, S. , C. Bonnerot, J. R. Drake, D. Choquet, W. Hunziker, J. -G. Guillet, P. Webster, C. Sautes, I. Mellman, and W. H. Fridman. 1992. Cytoplasmic domain heterogeneity and functions of IgG Fc receptors in B lymphocytes. *Science* 256:1808.

156. Hunziker, W. , T. Koch, J. A. Whitney, and I. Mellman. 1990. Fc receptor phosphorylation during receptor-mediated control of B-cell activation. *Nature* 345:628.

157. Engelhardt, W. , H. Gorczytza, A. Butterweck, H. Monkemann, and J. Frey. 1991. Structural requirements of the cytoplasmic domains of the human macrophage Fc gamma receptor IIa and B cell Fc gamma receptor IIb2 for the endocytosis of immune complexes. *Eur. J. Immunol.* 21:2227.

158. Ravetch, J. V. and B. Perussia. 1989. Alternative membrane forms of FcgammaRIII(CD16) on human natural killer cells and neutrophils. Cell type-specific expression of two genes that differ in single nucleotide substitutions. *J. Exp. Med.* 170:481.

159. Shen, L. , R. F. Graziano, and M. W. Fanger. 1989. The functional properties of FcgammaRI, II and III on myeloid cells: A comparative study of killing of erythrocytes and tumor cells mediated through the different Fc receptors. *Mol. Immunol.* 26:959.

160. Huizinga, T. W., K. M. Dolman, N. J. van der Linden, M. Kleijer, J. H. Nuijens, A. E. von dem Borne, and D. Roos. 1990. Phosphatidylinositol-linked FcRIII mediates exocytosis of neutrophil granule proteins, but does not mediate initiation of the respiratory burst. *J. Immunol.* 144:1432.

161. Huizinga, T. W., C. E. van der Schoot, C. Jost, R. Klaassen, M. Kleijer, A. E. von dem Borne, D. Roos, and P. A. Tetteroo. 1988. The PI-linked receptor FcRIII is released on stimulation of neutrophils. *Nature* 333:667.

162. Tosi, M. F. and M. F. Berger. 1988. Functional differences between the 40 kDa and 50-70 kDa IgG Fc receptors on human neutrophils revealed by elastase treatment and anti-receptor antibodies. *J. Immunol.* 141:2097.

163. Huizinga, T. W. J., F. van Kemenade, L. Koenderman, K. M. Dolman, A. E. von dem Borne, P. A. T. Tetteroo, and D. Roos. 1989. The 40-kDa FcgammaR (FcRII) on human neutrophils is essential for the IgG-induced respiratory burst and IgG-induced Phagocytosis. *J. Immunol.* 142:2365.

164. Looney, R. J., D. H. Ryan, K. Takahashi, H. B. Fleit, H. J. Cohen, G. N.

Abraham, and C. L. Anderson. 1986. Identification of a second class of IgG Fc receptors on human neutrophils. A 40 kilodalton molecule also found on eosinophils. *J. Exp. Med.* 163:826.

165. Tetteroo, P. A. T., C. E. van der Schoot, F. J. Visser, M. J. E. Bos, and A. E. von dem Borne. 1987. Three different types of Fcgamma Receptors on human leukocytes defined by workshop antibodies, FcRlow of neutrophils, FcRlow of K/NK lymphocytes and FcRII.. In Leukocyte Typing III; White Cell Differentiation Antigens. A. J. McMichael, editor. Oxford University Press, Oxford. 702

166. Huizinga, T. W. J., M. Kerst, J. H. Nuyens, A. Vlug, A. E. von dem Borne, D. Roos, and P. A. T. Tetteroo. 1989. Binding characteristics of dimeric IgG complexes to human neutrophils. *J. Immunol.* 142:2359.

167. Clark, M. R., L. Liu, S. B. Clarkson, P. A. Ory, and I. M. Goldstein. 1990. An abnormality of the gene that encodes neutrophil Fc receptor III in a patient with systemic lupus erythematosus. *J. Clin. Invest.* (in press)

168. Salmon, J. E., N. L. Brogle, J. C. Edberg, and R. P. Kimberly. 1991. Fc gamma receptor III induces actin polymerization in human neutrophils and primes phagocytosis mediated by Fc gamma receptor II. *J. Immunol.* 146:997.

169. Kimberly, R. P., J. W. Ahlstrom, M. E. Click, and J. C. Edberg. 1990. The glycosyl phosphatidylinositol-linked FcgammaRIII$_{PMN}$ mediates transmembrane signaling events distinct from FcgammaRII. *J. Exp. Med.* 171:1239.

170. Boros, P. , J. A. Odin, T. Muryoi, S. K. Masur, C. Bona, and J. C. Unkeless. 1991. IgM anti-FcgammaR autoantibodies trigger neutrophil degranulation. *J. Exp. Med.* 173:1473.

171. Nazruddin, B. , B. F. Duffy, J. Tucker, and T. Mohanakumar. 1992. Evidence for cross-regulation of FcgammaRIIIB (CD16) receptor-mediated signaling by FcgammaRII (CD32) expressed on polymorphonuclear cells. *J. Immunol.* 149:3702.

172. Stefanova, I. , V. Horejsi, I. J. Ansotegui, W. Knapp, and H. Stockinger. 1991. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science* 254:1016.

173. Frank, M. M., M. I. Hamburger, T. J. Lawley, R. P. Kimberly, and P. H. Plotz. 1979. Defective reticuloendothelial system Fc-receptor function in systemic lupus erythematosus. *N. Engl. J. Med.* 300:518.

174. Minuk, G. Y., M. Angus, C. M. Brickman, T. J. Lawley, M. M. Frank, J. H.

Hoofnagle, and E. A. Jones. 1985. Abnormal clearance of immune complexes from the circulation of patients with primary sclerosing cholangitis. *Gastroenterology.* 88:166.

175. Lawley, T. J., R. P. Hall, A. S. Fauci, S. I. Katz, M. I. Hamburger, and M. M. Frank. 1981. Defective Fc-receptor functions associated with the HLA-B8/DRw3 haplotype: studies in patients with dermatitis herpetiformis and normal subjects. *N. Engl. J. Med.* 304:185.

176. Meryhew, N. L., C. Shaver, R. P. Messner, and O. A. Runquist. 1991. Mononuclear phagocyte system dysfunction in murine SLE: abnormal clearance kinetics precede clinical disease. *J. Lab. Clin. Med.* 117:181.

177. Kimberly, R. P., A. Gibofsky, J. E. Salmon, and M. Fotino. 1983. Impaired Fc-mediated mononuclear phagocyte clearance in HLA-DR2 and MT1 positive healthy young adults. *J. Exp. Med.* 157:1698.

178. Hamburger, M. I., T. J. Lawley, R. P. Kimberly, P. H. Plotz, and M. M. Frank. 1982. A serial study of splenic reticuloendothelial system Fc receptor functional activity in systemic lupus erythematosus. *Arthritis. Rheum.* 25:48.

179. Frank, M. M., T. J. Lawley, M. I. Hamburger, and E. J. Brown. 1983. NIH Conference: Immunoglobulin G Fc receptor-mediated clearance in autoimmune diseases. *Ann. Intern. Med.* 98:206.

180. Sipos, A. , I. Sonkoly, L. Czirj:ak, S. Sipka, G. Szab:o, P. Boros, and G. Szegedi. 1988. Anti-granulocyte antibodies in immune neutropenia and autoimmune disorders. *Acta Paediatr. Hung.* 29:97.

181. Clarkson, S. B., R. P. Kimberly, J. E. Valinsky, M. D. Witmer, J. B. Bussel, R. L. Nachman, and J. C. Unkeless. 1986. Blockade of clearance of immune complexes by an anti-Fc gamma receptor monoclonal antibody. *J. Exp. Med.* 164:474.

182. Boros, P. , T. Muryoi, H. Spiera, C. Bona, and J. C. Unkeless. 1993. Autoantibodies directed against different classes of $Fc_{gamma}R$ are found in sera of autoimmune patients. *J. Immunol.* 150:2018.

183. Szegedi, A. , P. Boros, J. Chen, M. Kafina, C. Bona, and J. C. Unkeless. 1993. An FcRIII (CD16)-specific autoantibody from a patient with progressive systemic sclerosis. *Immunol. Lett.* 35:69.

184. Boros, P. , J. A. Odin, J. Chen, and J. C. Unkeless. 1993. Specificity and class distribution of $Fc_{gamma}R$-specific autoantibodies in patients with autoimmune

disease. *J. Immunol.* 152:302.

185. O'Shea, J. J., A. M. Weissman, I. C. S. Kennedy, and J. R. Ortaldo. 1991. Engagement of the natural killer cell IgG Fc receptor results in tyrosine phosphorylation of the zeta chain. *Proc. Natl. Acad. Sci. USA* 88:350.

186. Vivier, E. , P. Morin, C. O'Brien, B. Druker, S. F. Schlossman, and P. Anderson. 1991. Tyrosine phosphorylation of the FcgammaRIII(CD16):Zeta complex in human natural killer cells: Induction by antibody-dependent cytotoxicity but not by natural killing. *J. Immunol.* 146:206.

187. Einspahr, K. J., R. T. Abraham, B. A. Binstadt, Y. Uehara, and P. J. Leibson. 1991. Tyrosine phosphorylation provides an early and requisite signal for the activation of natural killer cell cytotoxic function. *Proc. Natl. Acad. Sci. USA* 88:6279.

188. Irving, B. A. and A. Weiss. 1991. The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell* 64:891.

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

190. Macintyre, E. A., P. J. Roberts, R. Abdul-Gaffar, K. O'Flynn, G. R. Pilkington, F. Farace, J. Morgan, and D. C. Linch. 1988. Mechanism of human monocyte activation via the 40-kDa Fc receptor for IgG. *J. Immunol.* 141:4333.

191. Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154:367.

192. Grynkiewicz, G. , M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440.

193. Ohta, S. , T. Taniguchi, M. Asahi, Y. Kato, G. Nakagawara, and H. Yamamura. 1992. Protein-tyrosine kinase p72syk is activated by wheat germ agglutinin in platelets. *Biochem. Biophys. Res. Commun.* 185:1128.

194. Burg, D. L., M. L. Harrison, and R. L. Geahlen. 1993. Cell cycle-specific activation of the PTK72 protein-tyrosine kinase in B lymphocytes. *J. Biol. Chem.* 268:2304.

195. Taniguchi, T. , T. Kobayashi, J. Kondo, K. Takahashi, H. Nakamura, J. Suzuki, K. Nagai, T. Yamada, S. Nakamura, and H. Yamamura. 1991. Molecular

cloning of a porcine gene syk that encodes a 72-kDa protein-tyrosine kinase showing high susceptibility to proteolysis. *J. Biol. Chem.* 266:15790.

196. Chan, A. C., B. Irving, J. D. Fraser, and A. Weiss. 1991. The TCR zeta chain associates with a tyrosine kinase and upon TCR stimulation associates with ZAP-70, a 70K Mr tyrosine phosphoprotein. *Proc. Natl. Acad. Sci. USA* 88:9166.

197. Korchak, H. M., L. B. Vosshall, K. A. Haines, C. Wilkenfeld, K. F. Lundquist, and G. Weissman. 1988. Activation of the human neutrophil by Calcium-mobilizing Ligands (II). *J. Biol. Chem.* 263:11098.

198. Hoffman, R. D. and D. M. Lane. 1992. Iodophenylarsine oxide and arsenical affinity chromatography: New probes for dithiol proteins. *J. Biol. Chem.* 267:14005.

199. Liao, K. , R. D. Hoffman, and M. D. Lane. 1991. Phosphotyrosyl turnover in insulin signaling. Characterization of two membrane-bound pp15 protein tyrosine phosphatases from 3T3-L1 adipocytes. *J. Biol. Chem.* 266:6544.

200. Garcia-Morales, P. , Y. Minami, E. Luong, R. D. Klausner, and L. E. Samelson. 1990. Tyrosine phosphorylation in T cells is regulated by phosphatase activity: Studies with phenylarsine oxide. *Proc. Natl. Acad. Sci. USA* 87:9255.

201. Wegener, A. M., F. Letourneur, A. Hoeveler, T. Brocker, F. Luton, and B. Malissen. 1992. The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules. *Cell* 68:83.

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

203. Bonnerot, C. , S. Amigorena, D. Choquet, R. Pavlovich, V. Choukroun, and W. H. Fridman. 1992. Role of associated gamma-chain in tyrosine kinase activation via murine Fc gamma RIII. *EMBO J.* 11:2747.

204. Phillips, N. E. and D. C. Parker. 1984. Cross-linking of B (lymphocyte) Fc gamma receptors and membrane immunoglobulin inhibits anti-immunoglobulin-induced blastogenesis. *J. Immunol.* 132:627.

205. Wilson, H. A., D. Greenblatt, C. W. Taylor, J. W. Putney, R. Y. Tsien, F. D. Finkelman, and T. M. Chused. 1987. The B lymphocyte calcium response to anti-Ig is diminished by membrane immunoglobulin cross-linkage to the Fc gamma receptor. *J. Immunol.* 138:1712.

206. McNeil, P. L., J. A. Swanson, S. D. Wright, S. C. Silverstein, and D. L.

Taylor. 1986. Fc-receptor-mediated phagocytosis occurs in macrophages without an increase in average [Ca++]i. *J. Cell Biol.* 102:1586.

207. Di Virgilio, F. , B. C. Meyer, S. Greenberg, and S. C. Silverstein. 1988. Fc receptor-mediated phagocytosis occurs in macrophages at exceedingly low cytosolic Ca2+ levels. *J. Cell Biol.* 106:657.

208. Hutchcroft, J. E., M. L. Harrison, and R. L. Geahlen. 1992. Association of the 72-kDa protein-tyrosine kinase PTK72 with the B cell antigen receptor. *J. Biol. Chem.* 267:8613.

209. Wange, R. L., A. N. Kong, and L. E. Samelson. 1992. A tyrosine-phosphorylated 70-kDa protein binds a photoaffinity analogue of ATP and associates with both the zeta chain and CD3 components of the activated T cell antigen receptor. *J. Biol. Chem.* 267:11685.

210. Hutchcroft, J. E., M. L. Harrison, and R. L. Geahlen. 1991. B lymphocyte activation is accompanied by phosphorylation of a 72-kDa protein-tyrosine kinase. *J. Biol. Chem.* 266:14846.

211. Weaver, C. T., J. T. Pingel, J. O. Nelson, and M. L. Thomas. 1991. CD8+ T-cell clones deficient in the expression of the CD45 protein tyrosine phosphatase have impaired responses to T-cell receptor stimuli. *Mol. Cell Biol.* 11:4415.

212. Koretzky, G. A., J. Picus, T. Schultz, and A. Weiss. 1991. Tyrosine phosphatase CD45 is required for T-cell antigen receptor and CD2-mediated activation of a protein tyrosine kinase and interleukin 2 production. *Proc. Natl. Acad. Sci. USA* 88:2037.

213. Iivanainen, A. V., C. Lindqvist T. Mustelin, and L. C. Andersson. 1990. Phosphotyrosine phosphatases are involved in reversion of T lymphoblastic proliferation. *Eur. J. Immunol.* 20:2509.

214. Pumiglia, K. M., L. F. Lau, C. K. Huang, S. Burroughs, and M. B. Feinstein. 1992. Activation of signal transduction in platelets by the tyrosine phosphatase inhibitor pervanadate (vanadyl hydroperoxide). *Biochem. J.* 286:441.

215. Levenson, R. M. and P. J. Blackshear. 1989. Insulin-stimulated protein tyrosine phosphorylation in intact cells evaluated by giant two-dimensional gel electrophoresis. *J. Biol. Chem.* 264:19984.

216. Bernier, M. , D. M. Laird, and M. D. Lane. 1987. Insulin-activated tyrosine phosphorylation of a 15-kilodalton protein in intact 3T3-L1 adipocytes. *Proc. Natl. Acad. Sci. USA* 84:1844.

217. Miller, J. , T. R. Malek, W. J. Leonard, W. C. Greene, E. M. Shevach, and R. N. Germain. 1985. Nucleotide sequence and expression of a mouse interleukin 2 receptor cDNA. *J. Immunol.* 134:4212.

218. Wigler, M. , A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin. 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells.. *Proc. Natl. Acad. Sci. USA* 76:1373.

219. Edberg, J. C. and R. P. Kimberly. 1992. Receptor Specific Probes for the Study of Fc-gamma Receptor Specific Function. *J. Immunol. Methods* 148:179.

220. Neville, D. M. 1971. Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system.. *J. Biol. Chem.* 246:6328.

221. Pelicci, G. , L. Lanfrancone, F. Grignani, J. McGlade, F. Cavallo, G. Forni, I. Nicoletti, T. Pawson, and P. G. Pelicci. 1992. A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* 70:93.

222. Wahl, M. I., S. Nishibe, P. G. Suh, S. G. Rhee, and G. Carpenter. 1989. Epidermal growth factor stimulates tyrosine phosphorylation of phospholipase C-II independently of receptor internalization and extracellular calcium. *Proc. Natl. Acad. Sci. USA* 86:1568.

223. McGlade, J. , A. Cheng, G. Pelicci, P. G. Pelicci, and T. Pawson. 1992. Shc proteins are phosphorylated and regulated by the v-Src and v-Fps protein-tyrosine kinases. *Proc. Natl. Acad. Sci. USA* 89:8869.

224. Lowenstein, E. J., R. J. Daly, A. G. Batzer, W. Li, B. Margolis, R. Lammers, A. Ullrich, E. Y. Skolnik, D. Bar Sagi, and J. Schlessinger. 1992. The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell* 70:431.

225. Shen, Z. , C. -T. Lin, and J. C. Unkeless. 1994. Correlations among tyrosine phosphorylation of Shc, p72syk, PLC-gamma1, and [Ca2+]i flux in FcgammaRIIA signaling. *J. Immunol.* 152:3017.

226. Meisenhelder, J. , P. G. Suh, S. G. Rhee, and T. Hunter. 1989. Phospholipase C-gamma is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. *Cell* 57:1109.

227. Kim, H. K., J. W. Kim, A. Zilberstein, B. Margolis, J. G. Kim, J.

Schlessinger, and S. G. Rhee. 1991. PDGF stimulation of inositol phospholipid hydrolysis requires PLC-gamma 1 phosphorylation on tyrosine residues 783 and 1254. *Cell* 65:435.

228. Nishibe, S. , M. I. Wahl, P. B. Wedegaertner, J. W. Kim, S. G. Rhee, G. Carpenter, and J. J. Kim. 1990. Selectivity of phospholipase C phosphorylation by the epidermal growth factor receptor, the insulin receptor, and their cytoplasmic domains [published erratum appears in Proc Natl Acad Sci U S A 1990 Apr;87(8):3253]. *Proc. Natl. Acad. Sci. USA* 87:424.

229. Morrison, D. K., D. R. Kaplan, S. G. Rhee, and L. T. Williams. 1990. Platelet-derived growth factor (PDGF)-dependent association of phospholipase C-gamma with the PDGF receptor signaling complex. *Mol. Cell Biol.* 10:2359.

230. Fallman, M. , D. P. Lew, O. Stendahl, and T. Andersson. 1989. Receptor-mediated phagocytosis in human neutrophils is associated with increased formation of inositol phosphates and diacylglycerol. *J. Clin. Invest.* 84:886.

231. Hidaka, H. , M. Inagaki, S. Kawamoto, and Y. Sasaki. 1984. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* 23:5036.

232. Cirillo, R. , M. Triggiani, L. Siri, A. Ciccarelli, G. R. Pettit, M. Condorelli, and G. Marone. 1990. Cyclosporin A rapidly inhibits mediator release from human basophils presumably by interacting with cyclophilin. *J. Immunol.* 144:3891.

233. Schreiber, S. L. and G. R. Crabtree. 1992. The mechanism of action of cyclosporin A and FK506. *Immunol. Today* 13:136.

234. Weiss, A. , G. Koretzky, R. C. Schatzman, and T. Kadlecek. 1991. Functional activation of the T-cell antigen receptor induces tyrosine phosphorylation of phospholipase C-gamma 1. *Proc. Natl. Acad. Sci. USA* 88:5484.

235. Hempel, W. M. and A. L. DeFranco. 1991. Expression of phospholipase C isozymes by murine B lymphocytes. *J. Immunol.* 146:3713.

236. Moraru, I. I., M. Laky, T. Stanescu, L. Buzila, and L. M. Popescu. 1990. Protein kinase C controls Fcgamma receptor-mediated endocytosis in human neutrophils. *FEBS Lett.* 274:93.

237. Yamane, Y. , M. Perez, R. Edelson, N. Agostino, and B. Pernis. 1991. Endocytosis of the TCR/CD3 complex and the class-I major histocompatibility complex in a human T cell line. *Cell Immunol.* 136:496.

238. Shin, J. , C. Doyle, Z. Yang, D. Kappes, and J. L. Strominger. 1990. Structural features of the cytoplasmic region of CD4 required for internalization. *EMBO J.* 9:425.

239. Ra, C. , K. Furuichi, J. Rivera, J. M. Mullins, C. Isersky, and K. N. White. 1989. Internalization of IgE receptors on rat basophilic leukemic cells by phorbol ester. Comparison with endocytosis induced by receptor aggregation. *Eur. J. Immunol.* 19:1771.

240. Shuler, R. L. and C. S. Owen. 1993. Initiation of Antigen Receptor Endocytosis and B Lymphocyte Activation Lie on Independent Biochemical Pathway. *Immunol. Cell. Biol.* 71 (Pt 1):1.

241. Lane, P. J., J. A. Ledbetter, F. M. McConnell, K. Draves, J. Deans, G. L. Schieven, and E. A. Clark. 1991. The role of tyrosine phosphorylation in signal transduction through surface Ig in human B cells. Inhibition of tyrosine phosphorylation prevents intracellular calcium release. *J. Immunol.* 146:715.

242. Stanley, J. B., R. Gorczynski, C. K. Huang, J. Love, and G. B. Mills. 1990. Tyrosine phosphorylation is an obligatory event in IL-2 secretion [published erratum appears in J Immunol 1991 May 1; 146(9):3260]. *J. Immunol.* 145:2189.

243. Veillette, A. , N. Abraham, L. Caron, and D. Davidson. 1991. The lymphocyte-specific tyrosine protein kinase p56lck. *Semin. Immunol.* 3:143.

244. Glaichenhaus, N. , N. Shastri, D. R. Littman, and J. M. Turner. 1991. Requirement for association of p56lck with CD4 in antigen-specific signal transduction in T cells. *Cell* 64:511.

245. Cooke, M. P., K. M. Abraham, K. A. Forbush, and R. M. Perlmutter. 1991. Regulation of T cell receptor signaling by a src family protein-tyrosine kinase (p59fyn). *Cell* 65:281.

246. Stein, P. L., H. M. Lee, S. Rich, and P. Soriano. 1992. pp59fyn mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell* 70:741.

247. Campbell, K. S., E. J. Hager, R. J. Friedrich, and J. C. Cambier. 1991. IgM antigen receptor complex contains phosphoprotein products of B29 and mb-1 genes. *Proc. Natl. Acad. Sci. U. S. A.* 88:3982.

248. Venkitaraman, A. R., G. T. Williams, P. Dariavach, and M. S. Neuberger. 1991. The B-cell antigen receptor of the five immunoglobulin classes. *Nature* 352:777.

249. Wange, R. L., S. N. Malek, S. Desiderio, and L. E. Samelson. 1993. Tandem SH2 domains of ZAP-70 bind to T cell antigen receptor zeta and CD3∈ from activated Jurkat T cells. *J. Biol. Chem.* 268:19797.

250. Kolanus, W. , C. Romeo, and B. Seed. 1993. T cell activation by clustered tyrosine kinases. *Cell* 74:171.

251. Desai, D. M., J. Sap, J. Schlessinger, and A. Weiss. 1993. Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. *Cell* 73:541.

252. Kishihara, K. , J. Penninger, V. A. Wallace, T. M. Kundig, K. Kawai, A. Wakeham, E. Timms, K. Pfeffer, P. S. Ohashi, M. L. Thomas, and et al. 1993. Normal B lymphocyte development but impaired T cell maturation in CD45-exon6 protein tyrosine phosphatase-deficient mice. *Cell* 74:143.

253. Hernandez Sotomayor, S. M., C. L. Arteaga, C. Soler, and G. Carpenter. 1993. Epidermal growth factor stimulates substrate-selective protein-tyrosine-phosphatase activity. *Proc. Natl. Acad. Sci. U. S. A.* 90:7691.

254. Plutzky, J. , B. G. Neel, and R. D. Rosenberg. 1992. Isolation of a src homology 2-containing tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA* 89:1123.

255. Shen, S. H., L. Bastien, B. I. Posner, and P. Chretien. 1991. A protein-tyrosine phosphatase with sequence similarity to the SH2 domain of the protein-tyrosine kinases. *Nature* 352:736.

256. Feng, G. S., C. C. Hui, and T. Pawson. 1993. SH2-Containing Phosphotyrosine Phosphatase as a Target of Protein-Tyrosine Kinases. *Science* 259:1607.

257. Vogel, W. , R. Lammers, J. huang, and A. Ullrich. 1993. Activation of a Phosphotyrosine Phosphatase by Tyrosine Phosphorylation. *Science* 259:1611.

258. Kazlauskas, A. , G. S. Feng, T. Pawson, and M. Valius. 1993. The 64-kDa protein that associates with the platelet-derived growth factor receptor beta subunit via Tyr-1009 is the SH2-containing phosphotyrosine phosphatase Syp. *Proc. Natl. Acad. Sci. U. S. A.* 90:6939.

259. Yi, T. and J. N. Ihle. 1993. Association of hematopoietic cell phosphatase with c-Kit after stimulation with c-Kit ligand. *Mol. Cell Biol.* 13:3350.

260. Villa Moruzzi, E. , S. Lapi, M. Prat, G. Gaudino, and P. M. Comoglio.

1993. A protein tyrosine phosphatase activity associated with the hepatocyte growth factor/scatter factor receptor. *J. Biol. Chem.* 268:18176.