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Fc receptor-mediated signal transduction: Roles of PI3K, PKC, calcium flux and tyrosine phosphorylation

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Fc Receptor-Mediated Signal Transduction: Roles of PI3K, PKC, Calcium Flux and Tyrosine Phosphorylation

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Abstract

Fc Receptor-Mediated Signal Transduction: Roles of PI3K, PKC, Calcium Flux and Tyrosine Phosphorylation

By Zhenhai Shen

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Receptors for the Fc domain of IgG (FcγR) on leukocytes mediate a pleiotropic response following crosslinking by immune complexes. Signaling events following crosslinking of B and T cell antigen receptors, FcϵRI, and FcγRs share common elements. In each, signaling is initiated by receptor crosslinking by antigen or immune complexes, and results in the activation of src family kinases and ZAP-70-related tyrosine kinases, which associate with members of the receptor complex. Subsequent events include phosphorylation on tyrosine of multiple cellular substrates, $[Ca^{2+}]_i$ transient and phagocytosis. Shc, Syk, phospholipase C-γ1 are rapidly phosphorylated on tyrosine residues upon crosslinking of human FcγRIIA transfected into $P388D_1$, a murine macrophage cell line. The $[Ca^{2+}]_i$ transient, activation of PI 3-kinase, as well as protein tyrosine phosphorylation, are necessary for both FcγRIIA and FcγRIIIB-mediated phagocytosis. Enhanced PI 3-kinase activity is associated with activated PLC-γ1 in transfected macrophages. Tyrosine phosphorylation of PLC-γ1 is

necessary but not sufficient for stimulated activity of the enzyme and release of IP3, its activity is dependent on PI 3-kinase activity. Crosslinking of FcγRIIA in macrophages results in activation of PKC-δ. Protein kinase C activation in neutrophils serves to blunt phagocytosis, particularly following FcγRIIIB activation, perhaps by inhibiting Ca^{2+} flux.

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INTRODUCTION

Receptors for the Fc domain of IgG (FcγR) on macrophages and neutrophils mediate 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. Co-aggregation of the B cell antigen receptor with FcγRs inhibit B cell differentiation (Phillips and Parker, 1985). The FcγR on placental syncytiotrophoblasts (Stuart et al., 1989) may be involved in transcytosis of immunoglobulin. The presence of high titers of anti-FcγR Ig (Boros et al., 1990; Sipos et al., 1988) has been reported in both human and mouse autoimmune disease.

Growth factor receptors are activated by ligand binding and subsequent dimerization. Similarly, FcγRs are triggered upon crosslinking or immobilization of the receptors by polyvalent immune complexes as a special case of dimerization. Reagents (such as anti-FcγR antibody) that crosslink the receptor by binding to epitopes other than those involved in the immunoglobulin Fc binding site are effective triggers (Ravetch and Kinet, 1991).

Signaling events mediated by antigen receptors on B and T cells (BCR and

TCR), FcεRI, and FcγRs share common elements, discussed in a recent review (Weiss and Littman, 1994). Key elements in transduction of signals by these receptors are the associated ζ (TCR), γ (FcεR and FcγRIIIA), and Igα and Igβ chains (BCR) which contain a conserved sequences first noted by Reth (Reth, 1989) and referred to as the antigen receptor activation motif (ARAM). Signal transduction by these receptors may differ in some respects since FcγRIIA has an ARAM with atypical spacing between the two Y-X-X-L motifs and FcγRIIIB has no cytoplasmic domain.

All FcRs except CD23 ($Fc_{\epsilon}RII$), are members of the Ig supergene family(Williams and Barclay, 1988). The nomenclature is summarized in Table 1. With the exception of huFcγRIIIB, which is anchored in the neutrophil plasma membrane by a glycan phosphatidyl inositol (GPI) moiety (Selvaraj et al., 1988), all FcγRs are class I membrane glycoproteins. Low avidity forms of membrane bound FcγRs contain two extracellular Ig-like regions, whereas high avidity forms contain three Ig-like regions. Assigning functions to individual FcγRs has been a challenging task since within a subclass receptors share immunologically indistinguishable extracellular domains and their cellular distributions overlap considerably.

The heterogeneity and genetic polymorphisms of human FcγRs

HuFcγRI (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, as determined by SDS-PAGE, of 72,000 (Anderson, 1982) (Table 1), which decreases after removal of N-linked carbohydrate to 40,000-50,000. In recent work, three highly homologous genes FcγRI genes (A, B, and C) have been identified, which encode for four huFcγRI transcripts, A1, B1, B2, and C1 (Ernst et al., 1992). The FcγRIA gene product, which was originally cloned using a eukaryotic expression shuttle vector (Allen and Seed, 1989), 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 (Hulett et al., 1991). 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 (Ernst et al., 1992). The transmembrane domain of the FcγRIA1 product, which appears to be the receptor previously identified with various antibodies (Ernst et al., 1992), is 21 residues and the cytoplasmic domain is short and highly charged (Allen and Seed, 1989; Sears et al., 1990). 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 (Allen and Seed, 1989). FcγR probably have redundant functions since several members of a Belgian family have a complete absence of huFcγRI expression on their peripheral blood monocytes (Ceuppens et al., 1988), 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 (Anderson, 1982). The anti-huFcγRII mAb IV.3 immunoprecipitates an antigen of about 40 kDa. HuFcγRII is a low affinity FcγR expressed widely on hematopoietic cells, including 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 IgG subclasses is the following: $IgG_1 = IgG_3 >> IgG_2 = IgG_4$ (Unkeless et al., 1992) (Table 2).

At least three genes, with homologous extracellular domains, but different transmembrane and cytoplasmic domains, encode FcγRII (Stuart et al., 1989; Brooks et al., 1989). HuFcγRIIA and FcγRIIC are preferentially expressed in neutrophils, while huFcγRIIB is preferentially expressed in lymphocytes. Monocytes express all three classes. HuFcγRIIA and huFcγRIIC are distinguished only by their signal

sequences, and probably are functionally identical. However, huFcγRIIB, which is homologous to the murine FcγRII, and also undergoes differential splicing (Ravetch and Kinet, 1991; Brooks et al., 1989), differs in sequence in the cytoplasmic domain. As a consequence of the absence of an ARAM sequence, FcγRIIB can not trigger a tyrosine kinase cascade(Alber et al., 1992; Daeron et al., 1992).

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 isotypic variation, two allelic forms of FcγRIIA with different affinity for murine IgG1 have been identified. The HR allotype, which has high affinity for murine IgG1, has Arg_{133} substituted for His_{133} (Clark et al., 1989). However, the FcγRIIA^{HR} allotype binds human IgG2 poorly while FcγRIIA^{LR} allotype interacts efficiently with human IgG2, suggesting that FcγRIIA may somehow regulate human IgG subclass production/turnover (Parren et al., 1992). The differences in affinity for human IgG2 between the HR and LR allotypes may be of functional importance, since PMN from individuals homozygous for FcγRIIA^{LR} phagocytosed IgG2-opsonized bacteria to a greater extent than PMN from individuals homozygous for FcγRIIA^{HR} (Bredius et al., 1993). Crosslinking FcγRIIA^{HR} transfected into mouse fibroblasts and/or Jurkat T cells by appropriate anti-CD3 mAbs led to IL6 production by accessory cells only when IgG1 anti-CD3 mAb was used (Duits et al., 1993).

Proteases may regulate FcγR functions *in vivo*. The binding of immune complexes and erythrocytes opsonized with murine IgG1 (EIgG1) to monocytes or

K562, a chronic myelogenous leukemia cell line that expresses only FcγRIIA, is increased following trypsin or pronase treatment (van de Winkel et al., 1989). This effect may be important in promoting clearance of opsonized particles at sites of inflammation, since neutrophil elastase and supernatants of PMN stimulated with chemotactic peptide have the same effect. Furthermore, monocyte binding of murine EIgG1 is inhibited by serine protease inhibitors (van de Winkel et al., 1990). Thus, there may be an obligatory proteolytic event required for binding by FcγRII. However, FcγRII receptor number and mobility are unchanged by proteolysis (van de Winkel et al., 1990; Tuijnman et al., 1990).

The generation of superoxide itself may result in activation of FcγRII (Gresham et al., 1990). Granulocyte-macrophage colony stimulating factor (GM-CSF) caused a three-fold increase in the formation of EIgG rosettes by eosinophils, which express only FcγRIIA, without alteration in the expression of FcγRIIA (Koenderman et al., 1993). Priming by GM-CSF or tumor necrosis factor-α (TNF-α) induced a two-fold enhancement in phagocytic index of EIgG by neutrophils, which was inhibited by the addition of superoxide dismutase. The increase in phagocytosis attributable to the production of superoxide was not seen in neutrophils from patients with chronic granulomatous disease (CGD), in which the respiratory burst is absent (Gresham et al., 1989).

HuFcγRIII (CD16)

Human FcγRIII (CD16) binds human IgG1 and IgG3 with Ka = 4 x 10^6 M^{-1} (Kurlander and Batker, 1982). There are two closely related genes, FcγRIIIA and FcγRIIIB (Ravetch and Perussia, 1989; Scallon et al., 1989). FcγRIIIA is a type I transmembrane protein, expressed on NK cells and macrophages. FcγRIIIB is tethered to the membrane surface via a GPI anchor, and is expressed exclusively on neutrophils. In mice, only one gene encodes FcγRIII, and there is no GPI-anchored form. The genes for huFcγRIII and muFcγRIII are located on the long arms of chromosome 1, closely clustered with the genes for FcγRII, FcγRI, and FcεRI (Qiu et al., 1990). Divergence of FcγRIIIA and FcγRIIIB genes may come from duplication and mutation of an ancestral FcγRIII gene. Differences between these two genes are confined to 9 nucleotide alterations, which account for 6 amino acid differences and the deletion of the 21 residues at C-terminus of FcγRIIIB (Ravetch and Perussia, 1989). The resulting FcγRIIIB precursor has a 4 residue cytoplasmic domain, which is processed in the endoplasmic reticulum to cleave the transmembrane domain and add the GPI anchor (Conzelmann et al., 1987). Expression of FcγRIIIB is only found on neutrophils, which also express FcγRIIA and FcγRIIC (CD32). FcγRIIIB is the predominant FcγR on PMN, with 100,000 - 300,000 molecules/cell, compared to 10,000 - 20,000 molecules/cell for FcγRII (A and C isoforms) (Huizinga et al., 1989).

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 (Selvaraj et al., 1989). A variable proportion of GPI-anchored proteins are PLC-resistant, due to palmitoylation of the inositol ring (Roberts et al., 1988), 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 (Selvaraj et al., 1989). There is, however, some evidence that the loss of all GPI-anchored proteins in PNH is not equal (Edberg et al., 1991), and that FcγRIIIB expression is maintained at low levels in the total absence of the GPI-anchored protein DAF (CD55) and CD59 (Edberg et al., 1991).

FcγRIIIB on human neutrophils has two allotypes, NA1 and NA2, and autoantibodies Na1 and Na2 are associated with juvenile neutropenia (Lalezari et al., 1986; Madyastha et al., 1982). The allotypes are co-dominantly expressed (Salmon et al., 1990). Substitution of Asn by Ser at position 63 and Asp by Asn at position 82 results in two additional glycosylation sites in the NA2 form (Ravetch and Perussia, 1989; Ory et al., 1989), which can be distinguished by mAbs CLB-GRAN 11 (NA1) and GRM 1 (NA2) (Trounstine et al., 1990). PMN from individuals homozygous for NA2 ingested fewer EIgG and E coated with concanavalin A (Salmon et al., 1990). FcγRIIIA always types as NA2. Studies of the monomeric IgG binding on large granule lymphocytes/NK cells (LGL/NK cells) have revealed a polymorphism in the level of FcγRIIIA expression (Vance et al., 1993).

Association of FcγRI and FcγRIIIA with γ and ζ chains

Recent work has shown that in addition to FcγRIIIA, FcγRI is also associated with a γ subunit (Ernst et al., 1993). 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 (Hibbs et al., 1989; Schoneich et al., 1992). 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 (Kurosaki et al., 1991). 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 negatively charged aspartate residue. Mutation of the aspartate to valine in the TM of huFcγRIIIAα lessened the dependence on γ chain for receptor expression (Kurosaki and Ravetch, 1989). This may explain why huFcγRI is not dependent on co-expression of the γ chain for surface expression (Ernst et al., 1992).

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 (Kinet, 1992). 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 (Keegan and Paul, 1992) and contain ARAM (antigen receptor activation motif), also termed as TAM (tyrosine-based activation motif) or ARH1 (antigen receptor homology 1) motifs (Samelson and Klausner, 1992; Clark et al., 1992) (Fig. 1). 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 (Huang et al., 1992). The expression of a polypeptide containing a single copy of the motif is sufficient for many aspects of MIRR signaling (Romeo et al., 1992). The evolution of multi-subunits 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. 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 (Keegan and Paul, 1992).

The association of the γ chain homodimer with human FcγRIIA is controversial. One group observed γ chain associated with all three types of FcγR in

human monocytes (Masuda and Roos, 1993), but another group, confirming association of γ chain with FcγRI and FcγRIIIA, failed to find co-precipitation with FcγRIIA (Ernst et al., 1993).

Fc receptor crosslinking activates tyrosine kinase

Cytoplasmic domain subunits of TCR, BCR, FCϵRI and FcγRIIIA complexes, termed multichain immune recognition receptors (MIRR) (Keegan and Paul, 1992), share a conserved antigen receptor activation motif (ARAM) with a dyad Y-X-X-L sequence in which two tyrosines are separated by 10-11 residues. The earliest event in MIRR signaling is the activation of at least one non-receptor tyrosine kinase. Mutational analysis of chimeric proteins revealed that an 18 residue ARAM would suffice to trigger signaling in T cells, and that both tyrosines in the conserved motif were crucial (Romeo et al., 1992). A primary event in signaling by MIRR is the activation of protein tyrosine kinase (PTKs). The kinetics of tyrosine phosphorylation precede phosphatidylinositol (PI) hydrolysis and $[Ca^{2+}]_i$ flux induced by MIRR ligation (Mustelin et al., 1990) and inhibitors of tyrosine kinase (genistein or herbimycin A) completely block the ability of TCR to stimulate PI turnover (Gold et al., 1990; Frank et al., 1992). 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 (Azzoni et al., 1993). Tyrosine kinase inhibitors inhibit Fc$_\epsilon$RI-

mediated degranulation (Benhamou et al., 1992) and FcγRIIIA-mediated $[Ca^{2+}]_i$ flux, PI turnover (Azzoni et al., 1993).

Two Src family members, Lck and Fyn, as well as another tyrosine kinase ZAP-70, have been implicated in TCR signal transduction (Straus and Weiss, 1992; Eiseman and Bolen, 1990; Chan et al., 1992). Lck associates with the cytoplasmic domain of both CD4 and CD8 T-cell surface glycoproteins. Lck may play a secondary role in TCR signaling but a primary role in thymocyte development (Molina et al., 1992). However, T cells from Fyn knockout mice did not proliferate following treatment with a combination of phorbol ester plus either anti-CD3 antibody or concanavalin (Appleby et al., 1992). ZAP-70 is essential for human T cell function. Mutation in the kinase domain of ZAP-70 resulted in an autosomal recessive form of severe combined immunodeficiency disease (SCID) and selective T cell deficiency disease in human (Elder et al., 1994; Chan et al., 1994; Arpaia et al., 1994).

Sequential interaction of TCR with two different tyrosine kinase was recently demonstrated. The two conserved tyrosine residues in the ARAM motif of ζ chain were initially phosphorylated by Lck, a Src family kinase, resulting in the recruitment of a second tyrosine kinase, ZAP-70, through its SH2 domain (Iwashima et al., 1994). Aggregation of chimeras of extracellular CD16 and intracellular Syk or ZAP-70 kinase sequences triggered $[Ca^{2+}]_i$ flux, whereas similar chimeras bearing a Src family kinase intracellular domain were not sufficient to initiate signaling (Kolanus et

al., 1993). However, the CD4 T cells from ZAP-70 deficient patients with selective T cell deficiency do flux $[Ca^{2+}]_i$, albeit at somewhat reduced levels, following TCR ligation (Arpaia et al., 1994). Thus, it appears that ZAP-70, although it plays an important role in TCR signaling, is not absolutely necessary. Alternatively, Syk may replace ZAP-70 during development in ZAP-70 deficient patients.

Src-like kinase also mediate FcγR signaling. Tyrosine kinase inhibitors resulted in the inhibition of FcεRI-mediated degranulation (Benhamou et al., 1992) and FcγRIIIA-mediated $[Ca^{2+}]_i$ flux, PI turnover, and NK cell ADCC (Alber et al., 1992). Neutrophil FcγRII stimulation led to the activation of Fgr (Hamada et al., 1993). FcγRII expressed on monocytic line TAP-1 cells was both physically and functionally associated with Src family kinase, Hck and Lyn (Ghazizadeh et al., 1994). A homologue of ZAP-70, Syk, was also activated upon crosslinking of FcγRIIA (Agarwal et al., 1993; Shen et al., 1994). Sequential interaction of a Src family kinase, Lyn, and Syk with β and γ subunits of FcεRI was reported (Jouvin et al., 1994), suggesting that amplification of signaling is necessary to achieve full activation.

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. Effector proteins implicated in FcγR signaling include Ras GTPase activating protein (GAP), phosphatidylinositol 3-kinase (PI 3-kinase), phospholipase C-γ1, and Shc. 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 kinase, contain this conserved domain (Koch et al., 1991), plays an important role in the intracellular responses to growth factor stimulation by binding to phosphotyrosine containing proteins (Margolis, 1992). Different SH2 regions have specificity for binding to particular tyrosine phosphorylated sites, as has been demonstrated for the binding of p85 subunit of PI 3-kinase, GTPase activating protein (GAP) and PLC-γ to activated growth factor receptor (Fantl et al., 1992; Kashishian et al., 1992; Rotin et al., 1992). 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* (Fukui et al., 1991a; Mayer et al., 1992; Matsuda et al., 1992).

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 (Ren et al., 1993), 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 (Duchesne et al., 1993). 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 (Clark et al., 1992).

$Fc_\epsilon RI$ 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 (Li et al., 1992). The receptor phosphorylation is rapidly reversible upon receptor disengagement (Paolini et al., 1991). $Fc_\epsilon RI$ crosslinking results in tyrosine phosphorylation of PLC-γ1 (Li et al., 1992). Activation of PLC-γ1 is known to occur through phosphorylation of multiple tyrosine residues without the participation of G proteins (Nishibe et al., 1990a). Serine phosphorylation of PLC-γ1 by PKA (cAMP-dependent kinase) and PKC may serve to modulate the interaction of the enzyme with tyrosine kinase or phosphatases (Park et al., 1992). 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 (Paolini and Kinet, 1993).

In addition to 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 $Fc_{\epsilon}RI$ (Kennerly, 1990). 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 PA phosphohydrolase conversion of PA to DAG) resulted in the inhibition of $Fc_{\epsilon}RI$-mediated release of histamine and arachidonate metabolites (Lin et al., 1991). PA, produced by PLD action, has also been implicated in triggering of the neutrophil respiratory burst in response to chemotactic peptide (Rossi et al., 1990; Billah et al., 1989). The role of PLD in $Fc\gamma R$ signaling remains to be determined.

The downstream targets following $Fc\gamma R$ crosslinking are less well known compared to the growth factor receptor system. Activation of $Fc\gamma RIIIA$ expressed in the natural killer (NK) cells led to the tyrosine phosphorylation of PLC-γ1, PLC-γ2 and p56lck (Azzoni et al., 1993; Ting et al., 1992) and activation of PI 3-kinase (Kanakaraj et al., 1994). Hu$Fc\gamma RI$ and hu$Fc\gamma RII$ upon activation mediate the tyrosine phosphorylation of PLC-γ1. Pretreatment with herbimycin A abolished this phosphorylation as well as phosphatidylinositol (PI) turnover (Liao et al., 1992).

GTPase activating protein (GAP) is a 120kD protein that directly interacts with cellular ras protein and enhances the intrinsic GTPase activity of Ras, thereby acting

as a negative regulator by converting Ras from the active GTP-bound form to the inactive GDP-bound form (Trahey and McCormick, 1987). Molecular cloning of GAP cDNA shows that GAP contains two SH2 domains and one SH3 domain at its N-terminus and a GTPase-activating domain at its C-terminus (Trahey et al., 1988; Vogel et al., 1988). GAP is phosphorylated on tyrosine residues and is physically associated with the PDGF receptor, Raf-1, PLC-γ1 and PI 3-kinase following PDGF treatment of cells expressing wild type PDGF receptor (Kaplan et al., 1990). However, in cells transformed by activated c-Ha-*ras*, GAP failed to associate with the receptor or increase its phosphotyrosine content in response to PDGF (Kaplan et al., 1990). This result suggests that GAP is crucial for signal transduction of PDGF receptor and for regulation of the Ras activity.

Two GAP associated proteins have been identified, p190 and p62. The carboxy-terminal portion of p190 protein sequence is homologous to proteins that possess an intrinsic GAP activity, and is also related to a transcriptional repressor of the glucocorticoid receptor, GRF-1 (Settleman et al., 1992). The formation of the GAP-p190 complex is dependent on tyrosine phosphorylation and shows reduced GAP activity (Beham et al., 1988). p62 shows sequence similarity to a putative hnRNP protein, GRP33 (Wong et al., 1992). In v-*src* transformed Rat-2 cells, a minor fraction of GAP associates with the highly phosphorylated p62 to form a complex that is localized at the plasma membrane. These data suggest that GAP-p62 complex plays a role in some aspect of mRNA processing or utilization and that this role may be

regulated by tyrosine phosphorylation, and indirectly by Ras.

The role of GAP domains in signal transduction becomes more intriguing with the observation that NF1 (neurofibromatosis type 1 susceptibility gene) and bcr (break point region), have GAP activity (DeClue et al., 1992; Xu et al., 1990b; Xu et al., 1990a). Cells derived from malignant NF1 tumor express a very low level of neurofibromin (Basu et al., 1992), suggesting that neurofibromin is a tumor suppressor, as loss of function contributes to tumor growth.

Another signaling molecule, phosphatidylinositol (PI) 3-kinase, has received much attention recently. It is a heterodimer, consisting of 110 kD (p110) catalytic subunit and 85 kD (p85) regulatory subunit. The PI 3-kinase phosphorylates the D-3 position of phosphatidylinositol (PI) forming PI 3-phosphate [PI(3)P], PI 3,4-bisphosphate [$PI(3,4)P_2$] and PI 3,4,5-trisphosphate [$PI(3,4,5)P_3$] (Auger et al., 1989; Whitman et al., 1988). The products of PI 3-kinase may be involved in the control of cellular growth and metabolism as a novel second messager (Auger et al., 1989). The p85 regulatory subunit contains two SH2 domains and one SH3 domain. A fragment of p85 containing the region between the two SH2 domains binds to p110 (Klippel et al., 1993). The SH2 domains of p85 direct the interactions of PI 3-kinase and phosphotyrosine-containing proteins (McGlade et al., 1992; Klippel et al., 1992) with the Y-X-X-M motif (Fantl et al., 1992; Kazlauskas et al., 1992). However, in Jurkat T cells, competition experiments with peptides suggested that PI 3-kinase binds to the first ARAM motif (Y-X-X-L) of the ζ chain.

The exact roles of PI 3-kinase are not fully understood. In addition to lipids, proteins may also be substrates for PI 3-kinase. PI 3-kinase contains intrinsic serine kinase activity that phosphorylates IRS-1, a critical molecule involved in insulin receptor signaling (Lam et al., 1994). *VPS*34 gene product, a yeast p110 homologue containing intrinsic PI 3-kinase activity, is essential for protein sorting, suggesting a possible role of PI 3-kinase in mammalian protein sorting (Schu et al., 1993). It is well established that PI 3-kinase is involved in growth factor receptor signaling (Cantley et al., 1991). Increasing evidence indicates that PI 3-kinase participates in MIRR signaling. The proline-rich region (residues 84 to 99) of p85 was associated with SH3 domain of activated Lyn and Fyn as a result of ligation of B cell antigen receptor, leading to increased PI 3- kinase activity (Pleiman et al, 1994). CD28, the T cell antigen required for T cell proliferation, bound to PI 3-kinase via its cytoplasmic Y-M-X-M motif (Prasad et al., 1994). Recently, it was reported that activation of FcγRII in U937 cells and FcγRIIIA in NK cells led to enhanced PI 3-kinase activity (Ninomiya et al., 1994; Kanakaraj et al., 1994). Thus, the interaction between PI 3-kinase and other signaling molecules may be biologically important, and its exact signaling mechanism needs further elucidation.

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 (Indik et al., 1991; Odin et al., 1991), but was unable to do so in CHO fibroblasts (Odin et al., 1991). HuFcγRIIB (b1 form), which does not contain the MIRR motif, was unable to mediate phagocytosis in COS cells (Tuijnman et al., 1992), nor does it trigger $[Ca^{2+}]_i$ flux (Kolanus et al., 1992). 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 b2, is able to mediate serotonin and TNF-α release when transfected into a rat basophilic leukemia cell line (RBL-2H3) (Latour et al., 1992). Identical results were obtained when $[Ca^{2+}]_i$ flux, phosphoinositide hydrolysis, release of arachidonate metabolites, and protein tyrosine phosphorylation were assessed (Alber et al., 1992). Macrophages from γ-chain knockout mice, which only express FcγRII, were unable to phagocytose IgG-coated particles, showed defects in NK cell ADCC, mast cell allergic responses (Takai et al., 1994). Moreover, these mice had a significantly attenuated inflammatory response to immune complexes, suggesting that immune complex-triggered inflammation is initiated by cell bound Fc receptors (Sylvestre and Ravetch, 1994).

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 (Miettinen et al., 1989). Both forms of the receptor

will direct *Toxoplasma gondii* opsonized with IgG to lysosomes (Joiner et al., 1990). 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 (Amigorena et al., 1992). This 13-amino acid motif containing the ARAM sequence is both necessary and sufficient for FcγRII-mediated inhibition of B cell receptor signaling (Muta et al., 1994). 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 is inhibited by staurosporine and by prolonged culture of the cells in PMA, suggesting that protein kinase C is responsible (Hunziker et al., 1990).

Distinct regions of FcγRs regulate different receptor-mediated functions. A

deletion mutant of huFcγRIIA (Δ264) lacking the 17 carboxyl-terminal residues (including the ultimate YXXL of the motif) was still able to mediate phagocytosis of receptor-bound immune complexes, but failed to mediate $[Ca^{2+}]_i$ flux associated with activation of the wt FcγRIIA (Odin et al., 1991). 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 triggering $[Ca^{2+}]_i$ flux and mediating phagocytosis. 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 (Kolanus et al., 1992).

GPI anchored FcγRIIIB

FcγRIIIB, has a glycan phosphatidyl inositol (GPI) anchor at the carboxyl

terminus and is expressed exclusively on neutrophils (Scallon et al., 1989; Ravetch and Perussia, 1989). FcγRIIIB probably does not 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 (Shen et al., 1989). FcγRIIIB ligation has been demonstrated to trigger the release of hydrolases, but apparently cannot stimulate a respiratory burst (Huizinga et al., 1990). 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 (Huizinga et al., 1988). The blockade of FcγRII on neutrophils by mAbs inhibits the respiratory burst, which cannot be triggered by crosslinking of neutrophil FcγRIIIB alone (Huizinga et al., 1989). Cleavage of FcγRIIIB by elastase, leaving FcγRII intact, does not alter the superoxide burst (Tosi and Berger, 1988).

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 suggest that huFcγRIIIB is involved in the initial adherence of neutrophils to IgG-coated erythrocytes (Looney et al., 1986; Tetteroo et al., 1987). Likewise, huFcγRIIIB was essential for the binding of small immune complexes to neutrophils, whereas huFcγRII only weakly enhanced this binding (Huizinga et al., 1989). Yet, this essential binding role of huFcγRIIIB did not extend to large immune complexes. Neutrophils from patients with paroxysmal nocturnal hematuria, which only express

10% of normal levels of huFcγRIIIB, had normal metabolic responses to IgG-latex (Huizinga et al., 1989). A patient with SLE who did not express huFcγRIIIB on her neutrophils, was due to a probable deletion of the huFcγRIIB gene (Clark et al., 1990). The patient's neutrophils did have reduced ability to rosette IgG-coated E, as suggested by earlier studies of neutrophil function (Looney et al., 1986; Tetteroo et al., 1987). 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 (Salmon et al., 1991), and $[Ca^{2+}]_i$ flux (Kimberly et al., 1990; Lund Johansen et al., 1991). 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 (Boros et al., 1991). Similar observations have been made concluding that FcγRIIIB signaling was modulated by FcγRII (Nazruddin et al., 1992). Other evidence for interaction between neutrophil FcγRIIIB and FcγRII is the enhanced phagocytosis of anti-FcγRII Fab-coated erythrocytes following crosslinking of FcγRIIIB (Salmon et al., 1991). However, others have found that immunoprecipiates of GPI-anchored proteins co-precipitated src-family kinase (Stefanova et al., 1991). Thy-1, a surface T cell molecule with a GPI-anchor, is associated with Fyn, and this

association is dependent on the GPI linkage (Thomas and Samelson, 1992). Decay-accelerating factor (DAF or CD55), a GPI-anchored protein that protects cells from complement-mediated lysis by either preventing the formation of or dissociating C3 convertases, was shown to associate with both Lck and Fyn in DAF-transfected murine thymoma cells (Shenoy Scaria et al., 1992). This however, may be due to hydrophobic interactions between the myristoyl group on the kinase and the lipid moiety of the GPI anchor.

The molecules involved in FcγRIIIB signal transduction may be in close association with the receptor in specialized plasma membrane domains. Studies by fluorescence recovery after photobleaching, single particle tracking, and laser optical tweezers, provide evidence for constrained mobility of GPI-anchored proteins in the membrane. The diffusion constants of GPI-anchored proteins are much closer to conventional integral membrane proteins than lipids, and a substantial fraction of GPI-anchored proteins are immobile in the observed time scale (Duits et al., 1993; Edidin et al., 1991).

Other line of evidence also point to the presence of specialized plasma membrane domains. Remarkably, GPI-anchored proteins are insoluble in 1% Triton X-100, and can be isolated in detergent insoluble membranes that contain most of the sphingosine based lipids of the cell, but only a small percentage of the glycerol based phospholipids (Brown and Rose, 1992). These Triton X-100 membrane fractions are also highly enriched in caveolin (a component of caveolae thought to function in

vesicular traffic between the trans-Golgi and the cell surface), c-Yes (a member of Src family), annexin II, and both small and heterotrimeric GTP-binding proteins (Sargiacomo et al., 1993). The association of GPI-anchored proteins with the components of the Triton insoluble vesicles may be an artifact of the isolation or a biochemical isolation of domains in the plasma membrane. Evidence supporting the functional association between GPI-anchored proteins and caveolae has emerged: after crosslinking, GPI-anchored proteins co-localize with caveolae in living cells (Mayor et al., 1994). However, the exact relationship between the isolated Triton X-100 membranes and putative membrane domains containing GPI-anchored proteins remains to be seen.

Table 1-1. Properties of $Fc\gamma R$

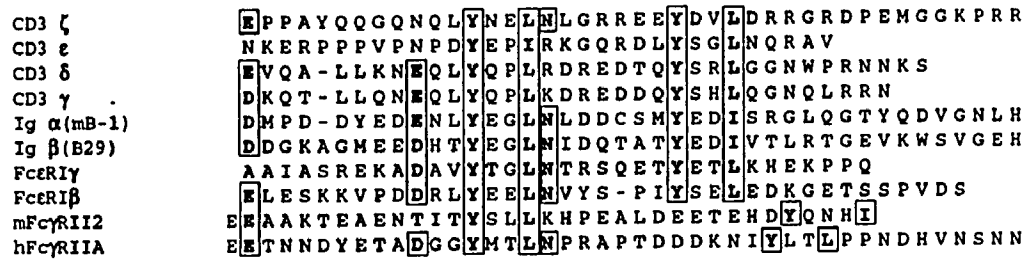
Receptor	Mol. Wt. (kDa)	Cell Distrib.	Affinity	mAbs
huFcγRIA,B,C	72	monocyte, macrophage, U937, HL60, INFγ treated neutrophils	high	32.2, 62, 22, 44,10.1, FR51
huFcγRIIA	40	U937, monocyte, neutrophil, platelet	low	IV.3, 2E1, KB56 41H.16
huFcγRIIB$_1$-B$_3$	40	B cell, U937, monocyte, neutrophil placenta	low	KB61, 2E1, IV.3
huFcγRIIC	40	B cell, U937, monocyte, neutrophil	low	KB61, 2E1 IV.3, 41H.16
huFcγRIIIA	50-70	monocyte, NK, macrophage	medium	3G8, B73.1 CLB-FcR-GRAN1 Leu-11a,b,c
huFcγRIIIB	50-70	neutrophil	low	same as above

Table 1-2. Ligand specificity of FcγRs

	Ligand specificity (IgG isotypes)	
Receptor	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

Figure 1-1. Alignment of ARAM sequences in receptors that activated by crosslinking

The ARAM (Antigen Receptor Activation Motif) motif has a dyad Y-X-X-L structure in the cytoplasmic domains of the multichain immune recognition receptor. Human FcγRIIA contains a modified ARAM sequence with wider space between two tyrosine residues.



Chapter 2

Materials and Methods

Reagents

Hybridoma IV.3 was obtained from American Type Culture Collection (Rockville, MD), and the mAb was purified from spent culture 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 (Lake Placid, NY), and was also provided as a generous gift by Dr. Thomas Roberts (Dana-Farber Cancer Institute, Boston, MA). Anti-Shc antiserum was provided as a generous gift by Dr. Tony Pawson (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Canada), and anti-PLC-γ1 antiserum was provided by Dr. Edward Skolnik (Department of Pharmacology, NYU Medical Center). Herbimycin A (Einspahr et al., 1991) was a kind gift by Dr. Yoshimada Uehara (National Institute of Health, Tokyo, Japan). Anti-CD18 mAb 1B4 $F(ab')_2$ was provided by Dr. Samuel Wright (Rockefeller University, NY). Alkaline phosphatase-conjugated secondary antibodies were from Organon-Technica Cappel (Malvern, PA). Unconjugated and biotinylated goat $F(ab')_2$ anti-mouse IgG were purchased from Jackson Immunoresearch (West Grove, PA). Protein A-agarose, nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3'-indolyphosphate *p*-toluidine salt (BCIP) were purchased from Pierce (Rockford, IL). Calphostin C, 3-aminopropyltriethoxysilane, BSA, glutaraldehyde, genestin, BrA23187, PMA and

Coomassie Blue R-250 were obtained from Sigma Chemical Co. (St. Louis, MO). Paraformaldehyde was from Polysciences, Inc. (Warrington, PA). Rhodamine-phalloidin, 1,2-bis-(t-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid acetoxymethylester (BAPTA-AM), and indo-1 acetoxymethylester (indo-1-AM) were purchased from Molecular Probes, Inc. (Eugene, OR).

Cells

$P388D_1$, a murine macrophage-like cell line, was transfected as described (2) with wild type huFcγRIIA cDNA or mutant huFcγRIIA cDNA (by Drs. Joseph A. Odin and Ching-Tai Lin, two former graduate student in the lab) . The transfected $P388D_1$ cell lines with wt (PW16) and mutant receptors (Δ264, Δ233, and Y252F) express 1.1-1.8 x 10^6 receptors per cell. The $P388D_1$ line was used as a transfection host to enable analysis of FcγRIIA mutants without competing wt FcγRIIA. Human neutrophils were isolated from buffy coat (Leukopac) preparations obtained from the Mount Sinai Hospital Blood Donor Center, and were purified using a two-step Ficoll gradient (English and Anderson, 1974). The neutrophils were collected from the 1.119 g/ml interface, washed, and resuspended in DME (buffered to pH 7.4 with 20 mM HEPES) containing 5% FCS.

Immune complex internalization assay

Transfected cells in suspension were incubated with mAb IV.3 Fab (1 μg/ml, 30 min, 4^{0}C), washed, and then incubated with goat F(ab')$_2$ anti-mouse IgG (2 μg/ml, 20 min, 4^{0}C) to crosslink the transfected FcγRIIA. The cells were then shifted to 37^{0}C for the indicated intervals and washed at 4^{0}C prior to labeling with FITC-conjugated rabbit F(ab')$_2$ anti-goat IgG (30 μg/ml, 30 min, 4^{0}C) as detailed previously (Odin et al., 1991).

$[Ca^{2+}]_i$ measurements

P388D$_1$ cells or neutrophils (6 x 10^6 cells/ml) were incubated in medium containing 5% FCS and 0.5 mM of indo-1-AM (Molecular Probes) for 30 min at room temperature. During this period, cells were also incubated with mAb 3G8 or IV.3 Fab fragments (0.1-2 μg/ml), and 1.2 μM calphostin C. After incubation, the neutrophils were washed and resuspended, at a density of 0.5-2 x 10^6 cells/ml, in a balanced salt solution (135 mM NaCl, 4.5 mM KCl, 5.6 mM glucose, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.4), and placed in a 1 cm pathlength quarts cuvette.

Ratiometric fluorescence measurements as a function of time were performed using a laboratory-modified, computer-controlled spectrofluorometer (SLM, Urbana, IL) capable of simultaneous digital acquisition at two wavelengths. Excitation at 355

nm and emission at 390 nm were selected by monochromators, while the long wavelength indo-1 emission band was observed through L-37 and Y-44 sharp cut glass filters (Hoya Corp. USA Optics Div. San Jose, CA). Neutral density filters were used to attenuate the excitation light intensity in order to minimize photobleaching of the probe. Crosslinking of FcγR was initiated by adding 35 μg/ml (final concentration) of goat F(ab')$_2$ anti-mouse IgG. As a positive conrol, and to assess cell viability, 100 nM FMLP was added to the cells following recovery from activation by FcγR crosslinking. After background correction, the fluorescence intensity values converted to $[Ca^{2+}]_i$ as previously described (Grynkiewicz et al., 1985). Ca^{2+}-saturated and Ca^{2+}-free ratio values were obtained by adding 10 μM A23187 to the cell suspensions, followed by 50 mM EGTA. A K_d of 300 nM for indo-1 at room temperature was used (Shuttleworth and Thompson, 1991).

IP3 assay

Cells were labelled with [^{3}H] myo-inositol (1 μCi/ml) (Amersham Life Sciences, Arlington Heights, IL) for 2 days in DMEM containing 5% FBS, collected by centrifugation, and washed with PBS containing 10 mM LiCl and 10 mM glucose. After incubation with mAb IV.3 Fab (2 μg/ml, 37^{0}C, 30 min), goat F(ab')$_2$ anti-mouse (40 μg/ml, 37^{0}C) was added. At intervals the cell suspension (100 μl) was pipetted into 1.2 ml of PBS containing 10 mM LiCl and 10 mM glucose prechilled in

a salt ice water bath. Following centrifugation for 10 sec, pellets were lysed in 1 ml of cold 10 mM formic acid for 1 hour. The extracted inositol phosphates were separated by an anion exchange column (AG 1-X4, 100-200 mesh, formate form, Bio-Rad) as described (Imai and Gershengorn, 1987).

Western blot analysis of tyrosine phosphorylation

Cells were collected from plates by gentle pipetting with DMEM, 2% FCS, 20 mM Hepes (pH 7.0) and dispensed at 2 x 10^6 cells/eppendorf tube. Gently pelleted cells were resuspended in 100 μl DMEM containing 20 mM Hepes, pH 7.0, and mAb IV.3 Fab (2 μg/ml, 30 min, 37^{0}C), washed, and stimulated by resuspension in 100 μl of the same medium containing goat F(ab')$_2$ anti-mouse IgG (40 μg/ml, 37^{0}C). In mock stimulations mAb IV.3 Fab was omitted. The cell suspension (100 μl) was diluted in 1.2 ml of PBS-2% FCS prechilled in a salt ice water bath. Following centrifugation for 10 sec, the supernatants were aspirated and lysis buffer (100μl, 0.5% NP-40, 0.1% sodium deoxycholate, 10% glycerol, 20 mM Na-PO_4, pH7.8, 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) was added. After the lysates were cleared by centrifugation (19,000 x g, 20 min, 4^{0}C) samples were electrophoresed on a 7-17% SDS-polyacrylamide gel (Neville, 1971), after which the proteins were transferred to nitrocellulose by electroelution. Blots

were blocked overnight at 4°C in TBS, 5% BSA (Sigma), 0.05% Tween-20, and 0.02% sodium azide. All subsequent steps were performed at room temperature. The blots were incubated with anti-phosphotyrosine mAb 4G10 (1 μg/ml, TBS, 1% BSA, 2hr). After three washes, bound 4G10 mAb was detected by incubation with alkaline phosphatase-conjugated goat $F(ab')_2$ anti-mouse IgG at 1:1000 dilution in TBS, 1% BSA, washed, and developed using NBT and BCIP (Pierce) as substrates.

Immunoprecipitation

Antiserum or purified IgG (2 μg IgG/sample) was added to 100 μl of detergent lysates (see above) from 2 x 10^6 cells and the mixture was incubated with nutation for 2 hr at 4°C. Protein A-agarose beads (25 μl, Pierce) were added and the mixture was incubated for 2 hr at 4°C. The beads were washed 5 times by centrifugation in lysis buffer, bound proteins were released by boiling in SDS sample buffer, and the eluted proteins were electrophoresed on SDS-PAGE. To immunoprecipitate FcγRIIA, biotinylated goat $F(ab')_2$ anti-mouse IgG was used in the initial activation of the cells. IV.3 Fab (1 μg/sample) was added to 100 μl lysate from 2 x 10^6 control or stimulated cells for 1 hr, and then biotinylated goat $F(ab')_2$ anti-mouse IgG (5 μg/sample) was added and nutated at 4°C for another hr. Streptavidin-conjugated agarose (Pierce) was used to collect the biotinylated complexes.

Preparation of covalently bound FcγRIIA

Proteins were covalently coupled to glass as described (Werb et al., 1989). Glass petri dishes or coverslips were acid washed with 20% H_2SO_4, neutralized with 0.1N NaOH, rinsed with water, dried, and derivatized with 3-aminopropyltriethoxysilane (Sigma) for 4 min at room temperature (rt). After rinsing with phosphate buffered saline (PBS), the dishes or coverslips were incubated with 0.25% glutaraldehyde (Sigma) for 30 min followed by incubation with 5 μg/ml of either goat $F(ab')_2$ anti-mouse IgG (Jackson Immunoresearch) or streptavidin in PBS for 1 hr at rt. The dishes or coverslips were rinsed with PBS, and cells (1 x 10^6/6 cm dish) suspended in medium containing 2% FCS were allowed to adhere for 30 min at 37°C. Finally, Fab of mAb IV.3 or 3G8 (or biotinylated Fab) was added to a final concentration of 1 μg/ml and incubated for varying intervals at 37°C.

Rhodamine phalloidin staining of F-actin

In order to determine the distribution of F-actin, coverslips with attached cells were fixed with 2% paraformaldehyde in PBS for 10 min at rt, and quenched with 50 mM NH_4Cl for 5 min. The cells were then permeabilized with 0.1% Triton X-100, rinsed with 3% normal horse serum, and stained with 0.8 μg/ml rhodamine-phalloidin for 30-45 min at rt.

Quantitative measurement of cell area

$P388D_1$ cells expressing wt and mutant FcγRIIA were plated on coverslips as detailed above, stimulated for different time intervals and fixed with 0.2% glutaraldehyde. They were then stained for 15 min with 0.2% Coomassie Blue R-250 dissolved in 20% methanol, followed by repeated rinsing in 5% acetic acid. The coverslips were quickly drained, air-dried, and mounted with glycerol for observation. Digital images were acquired using a Zeiss Axiovert microscope, a 10X, 0.25 NA Achrostigmat or a 40X, 0.75 NA water immersion objective and a cooled slow-scan CCD camera (OMA Vision, EG&G PARC, Princeton, NJ). In order to increase the image contrast, 560 nm transillumination light was selected by a 40 nm bandpass interference filter (Omega Optical, Inc., Brattleboro, VT). Automatic analysis of the digitized images was carried out using the Image-1 software package (Universal Imaging, West Chester, PA) and consisted of flatfield correction, noise reduction by application of a median filter, histogram thresholding and segmentation of the dark cells from the bright background, and measurement of the cell areas in terms of image pixels. The size of the area imaged by each pixel was determined by the use of an objective micrometer. Cell fragments and unresolved aggregates were excluded from further analysis on the basis of their sizes.

PI kinase and Diacylglycerol kinase assay

NP-40 lysates from macrophages stimulated in the absence or presence of wortmannin (10 nM, Biomol Research Laboratory, Inc., Plymouth Meeting, PA) as described above were immunoprecipitated with either anti-PLC-γ1 or anti-p85 regulatory subunit of PI3K IgG, and the immune complexes were collected with agarose conjugated-protein A at 4^{0}C. The beads were then washed sequentially with NP40 lysis buffer, PBS, LT buffer (0.5 M LiCl, 20 mM Tris-HCl, pH7.5), dH_2O, TEN buffer (20 mM Tris-HCl, pH7.5, 1 mM EDTA, 100 mM NaCl) and resuspended in 25 μl TGN buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 100 mM NaCl). 0.5 μl phosphatidylinositol (PI, 20 mg/ml in DMSO) (Avanti Polar Lipids, Inc., Alabastar, AL) or 0.5 μl of 1-stearoyl-2-arachidonoyl-sn-glycerol (20 mg/ml in DMSO, Sigma) (This was done by mixing the TGN and PI or DAG used for multiple samples to reach the final concentration described above) was added and the mixture was incubated at 25^{0}C for 10 min. To initiate the reaction, premixed 10 μCi γ-^{32}P-ATP (Amersham Life Sciences, Arlington Height, IL) and $MgCl_2$ (final concentration 20 mM) were added and mixture was further incubated at 25^{0}C for 10 min. The reaction was stopped by addition of stop solution (chloroform:methanol:11.6 N HCl = 100:200:2). The lipid metabolites were extracted by extra 150 μl chloroform, and speed-vacumn dried, resuspended in 20 μl chloroform and spotted onto silica gel 60 plates (Fisher, Springfield, NJ). The TLC was developed in

chloroform-methanol-30% ammonium hydroxide-H_2O (43:38:5:7). Radioactivity on the plates was visualized using a phosphor screen (Molecular Dynamics), and quantified using image quantification software IQ3.3 (Molecular Dynamics).

Chapter 3

Correlations Among Tyrosine Phosphorylation of Shc, Syk, PLC-γ1, and $[Ca^{2+}]_i$ Flux in FcγRIIA Signaling

Abstract

Tyrosine phosphorylation plays a critical role in FcγRIIA signaling. In a mouse macrophage cell line transfected with human FcγRIIA, 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 Syk. In addition, tyrosine phosphorylated FcγRIIA was coprecipitated with activated PLC-γ1. 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 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 through serine/threonine phosphorylation.

Activation of macrophage FcγRIIA by crosslinking results in internalization of the receptor complex, $[Ca^{2+}]_i$ flux, and tyrosine kinase activation. The cytoplasmic domain of FcγRIIA has a tyrosine kinase activation motif related to that found in subunits of the T and B cell antigen receptor complexes, FcεRI, and FcγRIIIA, all of which have been termed multichain immune recognition receptors (Keegan and Paul, 1992). Previous analysis of FcγRIIA cytoplasmic truncation mutants revealed that the 17-amino acid carboxy-terminal segment containing a Y-X-X-L motif is required for $[Ca^{2+}]_i$ flux but not for internalization of immune complexes. More extensive deletion of the cytoplasmic domain resulted in a receptor that mediated neither internalization, $[Ca^{2+}]_i$ flux, nor activation of tyrosine kinase (Odin et al., 1991).

Several signaling proteins including Shc (Pelicci et al., 1992) and PLC-γ1 (Wahl et al., 1989; Nishibe et al., 1990a) 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 (Pelicci et al., 1992). 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 kinase (McGlade et al.,

1992). In addition, tyrosine phosphorylated Shc forms a complex with a nonphosphorylated 23 kDa polypeptide encoded by the *grb2/sem5* gene (Clark et al., 1992; Lowenstein et al., 1992).

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 (Wahl et al., 1989; Meisenhelder et al., 1989; Kim et al., 1991). Tyrosine phosphorylation is correlated with the activation of PLC-γ1 and association with activated PDGF and EGF receptors (Nishibe et al., 1990b; Morrison et al., 1990). Serine phosphorylation of PLC-γ1 by either cAMP-dependent kinase or PKC may serve to modulate the interaction of the enzyme with tyrosine kinase and phosphatases.

Tyrosine kinase are intimately involved in signaling by multichain immune recognition receptors. Ligation of the receptor results in both the rapid tyrosine phosphorylation and activation of the non-receptor protein tyrosine kinase, Syk (Hutchcroft et al., 1991). However, Syk is not activated by PMA or Ca^{2+} ionophores (Yamada et al., 1991). We have now examined the correlations among tyrosine phosphorylation of Syk, Shc, PLC-γ1 and calcium mobilization. The potential role of protein kinase C has also been examined in P388D_1 cell lines expressing wild type (wt) and mutant FcγRIIA.

Results

Shc is phosphorylated on tyrosine residues upon crosslinking FcγRIIA

Crosslinking of wild type FcγRIIA in P388D$_1$ cells (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 1, lanes 1,2). Binding of mAb IV.3 Fab alone, or addition of the goat F(ab')$_2$ anti-mouse IgG without IV.3 Fab had no effect. The mammalian *Shc* gene encodes two overlapping proteins of 47 and 52 kDa, with a carboxy-terminal SH2 domain and a region with homology to collagen but no identifiable catalytic domain (Pelicci et al., 1992). To determine if the 52,000 M_r protein is Shc, detergent lysates of cells before and after FcγRIIA activation were immunoprecipitated with anti-Shc sera followed by immunoblotting with anti-phosphotyrosine mAb 4G10. Proteins of 47,000, 52,000, and 66,000 M_r were seen 1-2 min after activation of FcγRIIA in anti-phosphotyrosine immunoblots from PW16 cells. The 66,000 M_r protein is from a related gene not yet cloned (Pelicci et al., 1992). However, crosslinking the FcγRIIA deletion mutants Δ264 and Δ233 did not result in tyrosine phosphorylation of Shc (Fig. 1, lanes 12-14, 17-19). The Y252F FcγRIIA mutant, upon crosslinking, results in a small amount of Shc phosphorylation (Fig. 1, lanes 22-24). It is interesting to note that a protein with M_r 130 kDa was co-immunoprecipitated with Shc. A similar phosphorylation pattern of Shc was

observed upon FcγRIIA activation of U937 cells, a human promonocytic line that expresses endogenous huFcγIIA (Fig. 2A) as well as human neutrophils (Fig. 2B).

Tyrosine phosphorylation of PLC-γ1 upon FcγRIIA activation

PLC-γ1, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into the second messengers diacylglycerol and 1,4,5-inositol trisphosphate (IP3), is activated by tyrosine phosphorylation (Nishibe et al., 1990a; Kim et al., 1991). In addition, the activation of PDGF receptor (Morrison et al., 1990) and EGF receptor (Rotin et al., 1992) results in the formation of a complex between the activated receptors and PLC-γ1 that is dependent on SH2 domain interaction with phosphotyrosine. In agreement with earlier work (Liao et al., 1992), activation of FcγRIIA resulted in tyrosine phosphorylation of PLC-γ1 visible by 1 min (Fig. 3, lanes 1-6). No tyrosine phosphorylation of PLC-γ1 was detected in Δ264 and Y252F FcγRIIA mutants (Fig. 3, lanes 7-14), neither of which trigger $[Ca^{2+}]_i$ flux. Immunoprecipitation of PLCγ-1 also resulted in co-precipitation of a tyrosine-phosphorylated protein with characteristic mobility (M_r 40,000) of FcγRIIA expressed in $P388D_1$ cells (Fig. 3, lane 6,16). We do not see a band with the mobility of PLC-γ1 in immunoprecipitates of FcγRIIA.

Tyrosine phosphorylation of Syk upon FcγRIIA activation

The rapidly phosphorylated species of 72,000 M_r is the same size as Syk (also called PTK72) (Ohta et al., 1992), a Syk-family kinase activated by ligation of the B cell antigen receptor (Burg et al., 1993), the mast and basophil FcεRI (Hutchcroft et al., 1992a), and platelets following activation with wheat germ agglutinin (Yamada et al., 1991). After crosslinking of transfected FcγRIIA, immunoblotting of an anti-Syk immunoprecipitate for phosphotyrosine confirmed the presence of tyrosine-phosphorylated Syk (Fig. 4A). However, Immunoprecipitation with anti-Syk showed the absence of tyrosine-phosphorylation of Syk following activation of Δ264 and Y252F cells (Fig. 4B, C).

Effects of PMA

Since phagocytosis by FcγR in neutrophils is associated with production of IP3 and diacylglycerol, resulting in PKC activation (Fallman et al., 1989), 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 (data not shown). Pretreatment of PW16 cells with 1 μM and 25 μM of the protein kinase inhibitor, H8 (Hidaka et al., 1984), 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 (data not shown).

Pretreatment with PMA decreased the level of $[Ca^{2+}]_i$ flux following FcγRIIA crosslinking in a dose-dependent manner (not shown). Tyrosine phosphorylation of Shc was also dramatically inhibited by PMA pretreatment (Fig. 5A). However, PMA had no effect on the level of tyrosine phosphorylation of either PLC-γ1 or Syk (Fig. 5B, C). PMA pretreatment resulted in elevation of the basal phosphotyrosine level of a band with M_r of 74,000. The phosphotyrosine content of the band was further increased in intensity by FcγRIIA stimulation of PMA pretreated cells, and this protein was subsequently shown to be the PKC-δ (see Chapter 4 for results and discussion).

IP3 production correlates with $[Ca^{2+}]_i$ flux

Tyrosine phosphorylation of Shc was correlated with calcium mobilization, and both were inhibited by PMA. Since there was no effect of PMA on the tyrosine phosphorylation level of PLC-γ1, but a decrease in the flux of $[Ca^{2+}]_i$, we examined the level of the second messenger IP3 produced by cleavage of PIP2. IP3 concentration rose by 1 min after activation of FcγRIIA (Fig. 6). Moreover, PMA pretreatment of PW16 cells resulted in inhibition of IP3 production after receptor activation (Fig. 6), which was consistent with the inhibition of $[Ca^{2+}]_i$ flux.

Discussion

Activation by crosslinking of FcγRIIA expressed in macrophages results in phosphorylation on tyrosine of a set of cellular substrates, $[Ca^{2+}]_i$ flux, and internalization of complexes. We have chosen to analyze FcγRIIA function in the murine macrophage cell line $P388D_1$, but it is likely that these events also occur in human macrophages, since the tyrosine phosphorylation patterns of U937 cells are remarkably similar to the $P388D_1$ cells transfected with human FcγRIIA following receptor crosslinking (Fig. 2A). We have been interested in dissecting which substrates for cellular kinase are needed for specific effector functions. $P388D_1$ cells expressing Δ264 FcγRIIA can internalize immune complexes but cannot phagocytose large particles nor mediate a $[Ca^{2+}]_i$ flux. The Y252F FcγRIIA mutant cannot mediate a $[Ca^{2+}]_i$ flux, and is severely compromised in internalization of immune complexes (unpublished results).

The $[Ca^{2+}]_i$ flux observed in B cells, T cells, and macrophages upon crosslinking of antigen receptors (Weiss et al., 1991; Hempel and DeFranco, 1991), FcγRI, and FcγRII (Liao et al., 1992) 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 (Park et al., 1992). 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.

We found that activation by crosslinking of FcγRIIA expressed in $P388D_1$ cells led to rapid and transient tyrosine phosphorylation of Shc, thought to be an adaptor that couples tyrosine kinase to downstream targets that lack SH2 domain. Shc is associated with, and is phosphorylated on tyrosine by activated EGF receptor (Pelicci et al., 1992), and Shc may be an *in vivo* substrate for the v-Src and v-Fps non-receptor tyrosine kinase in Rat-2 cells transformed by v-*src* or v-*fps* (McGlade et al., 1992). Although PMA had no effect on tyrosine phosphorylation of PLC-γ1, pretreatment with PMA did inhibit the tyrosine phosphorylation of Shc.

Activation of protein tyrosine kinase 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 kinase (Eiseman and Bolen, 1990), stimulation of FcγRIIIA results in lck activation (Azzoni et al., 1993), and neutrophil FcγRII stimulation activates the fgr kinase (Hamada et al., 1993). These

kinase then activate a second tyrosine kinase, Syk, which participates in activation of B cells by the antigen receptor (Hutchcroft et al., 1992b), RBL-2H3 rat basophilic leukemia cells by the high avidity FcεRI (Hutchcroft et al., 1992a), and platelets activated by lectins (Ohta et al., 1992). A closely related kinase, ZAP-70, is activated in T cells (Chan et al., 1992). Current models for signal transmission by the TCR complex postulate an initial activation of a Src kinase, either Lck or Fyn, which phosphorylates the dyad tyrosines in the ARAM motif. this leads to binding, and activation of ZAP-70, which is the primary kinase mediating the phosphorylation of other cellular substrates, such as PLC-γ1 and Shc (Iwashima et al., 1994; Weiss and Littman, 1994; Irving et al., 1993). Our results confirm recent work demonstrating that Syk was activated upon crosslinking FcγRIIA (Agarwal et al., 1993). We have also found that Syk was not tyrosine phosphorylated in mutants that failed to trigger $[Ca^{2+}]_i$ flux, indicating that an intact carboxyl-terminal Y-X-X-L motif is required for the tyrosine phosphorylation and activation of Syk as well as tyrosine phosphorylation of Shc and PLC-γ1. Since the major tyrosine phosphorylated proteins missing in the mutants were PLC-γ1 and Shc, the above results argue that 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 (Moraru et al., 1990) has been reported, and PMA has also been shown to induce the internalization of the TCR/CD3 complex (Yamane et al., 1991), CD4

(Shin et al., 1990), and the IgE receptor (Ra et al., 1989), but not surface immunoglobulin on murine B lymphocytes (Shuler and Owen, 1993). 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 Syk induced by FcγRIIA activation, it did inhibit Shc phosphorylation to roughly the same extent as $[Ca^{2+}]_i$ flux was inhibited. 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 (Park et al, 1992). The decrease in the extent of IP3 production mediated by PMA 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. 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.

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Figure 3-1. Tyrosine phosphorylation of Shc following FcγRIIA stimulation. Cells expressing wt (lanes 1-9) and mutant (lanes 10-24) FcγRIIA were stimulated by crosslinking the FcγRIIA, lysed in detergent, and the cleared lysates were immunoprecipitated with anti-Shc serum. Preimmune serum was used as control. After SDS-PAGE, proteins were transferred to nitrocellulose, and stained with anti-phosphotyrosine mAb 4G10.

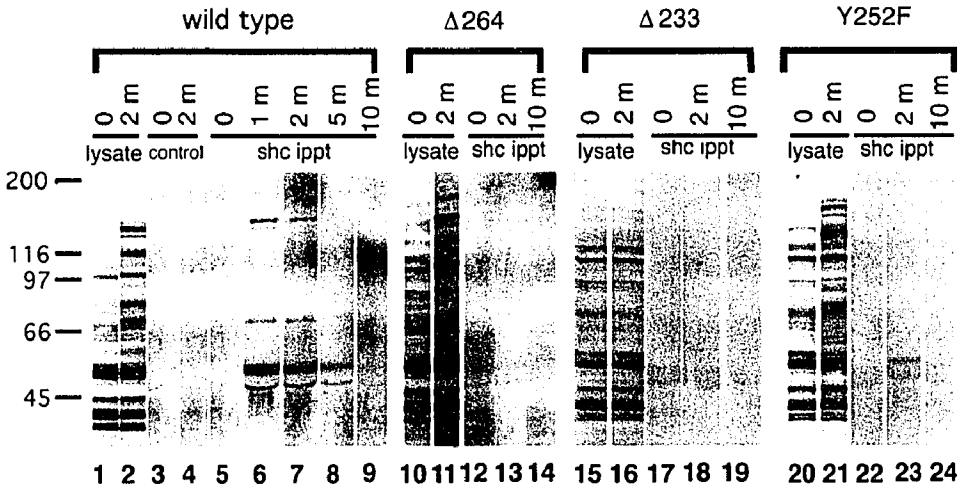


Figure 3-2. Tyrosine phosphorylation of Shc following receptor activation in **(A)** U937 cells and **(B)** neutrophils. Experimental procedures were as described in Fig.1.

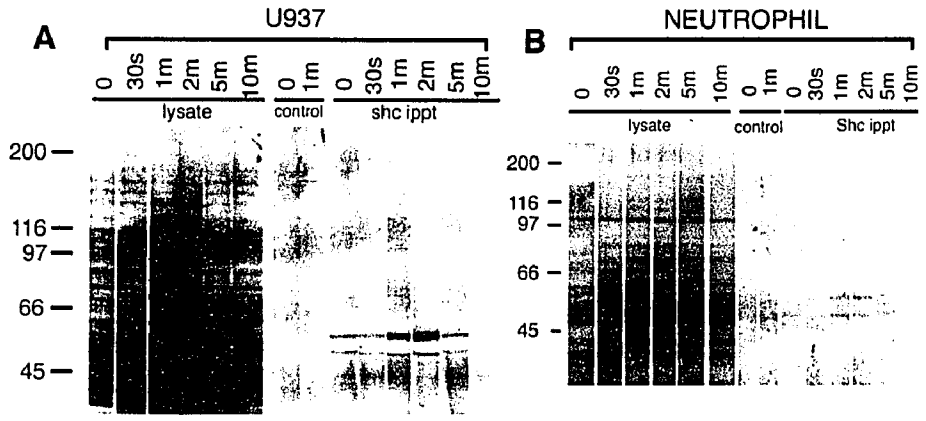


Figure 3-3. Tyrosine phosphorylation of PLC-γ1 following crosslinking FcγRIIA (lanes 1-6) and FcγRIIA mutants (lanes 7-14) expressed in $P388D_1$ cells. Cell lysates were immunoprecipitated with either anti-PLC-γ1 (lanes 3-6,9,10,13,14) or mAb IV.3 (lanes 15, 16), subjected to SDS-PAGE, transferred to nitrocellulose, and stained with anti-phosphotyrosine mAb 4G10.

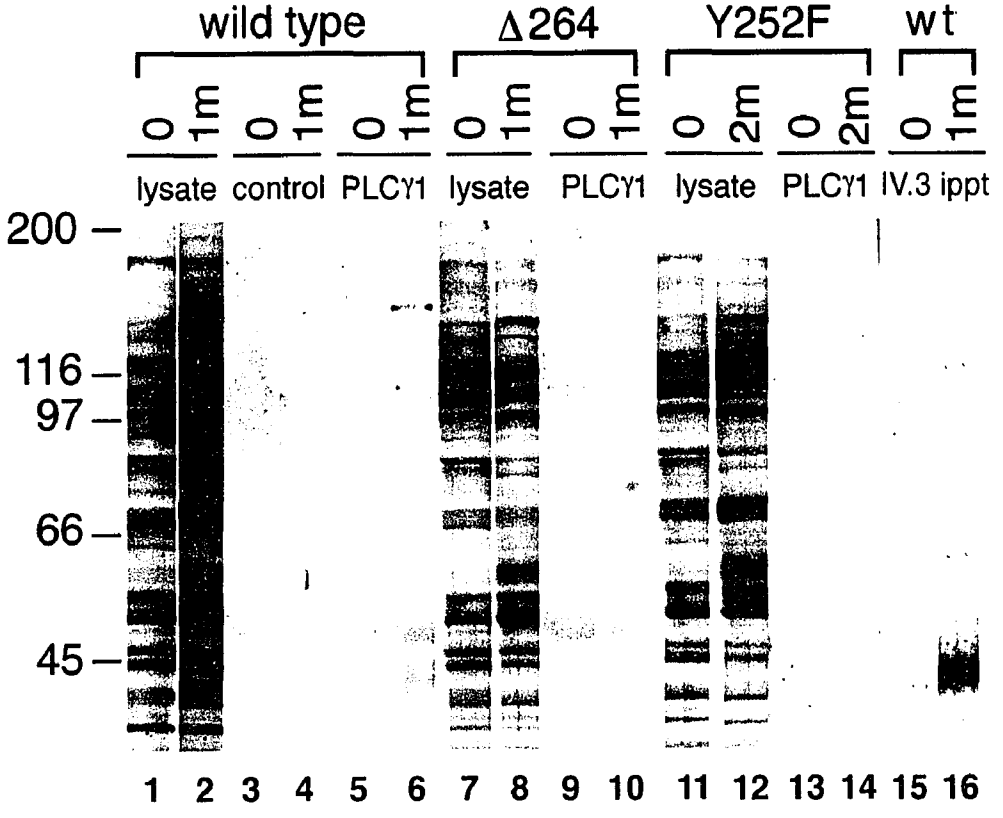


Figure 3-4. tyrosine phosphorylation Syk upon crosslinking of FcγRIIA. Control and stimulated lysates were immunoprecipitated with anti-Syk antibody and immunoblotted for phosphotyrosine. **(A)** wt cells; **(B)** Δ264 deletion mutant; **(C)** Y252F point mutant.

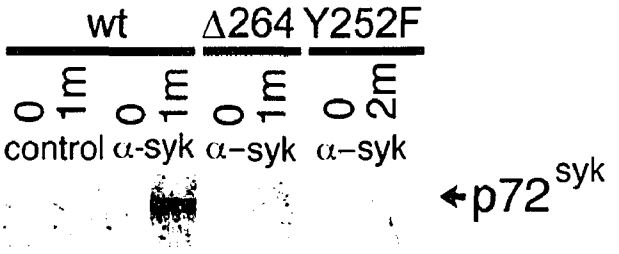


Figure 3-5. Analysis of PMA treatment on tyrosine phosphorylation of (A) Shc, (B) PLC-γ1 and (C) Syk. Cells were treated with varying concentration of PMA for 15 min before crosslinking FcγRIIA as described in Methods. Preimmune sera were used as controls for the anti-Shc, anti-PLC-γ1 and anti-syk sera.

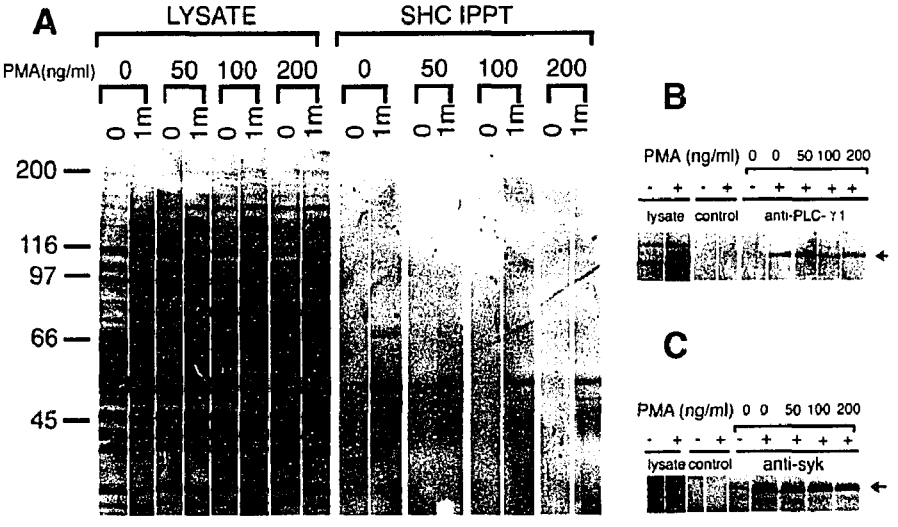
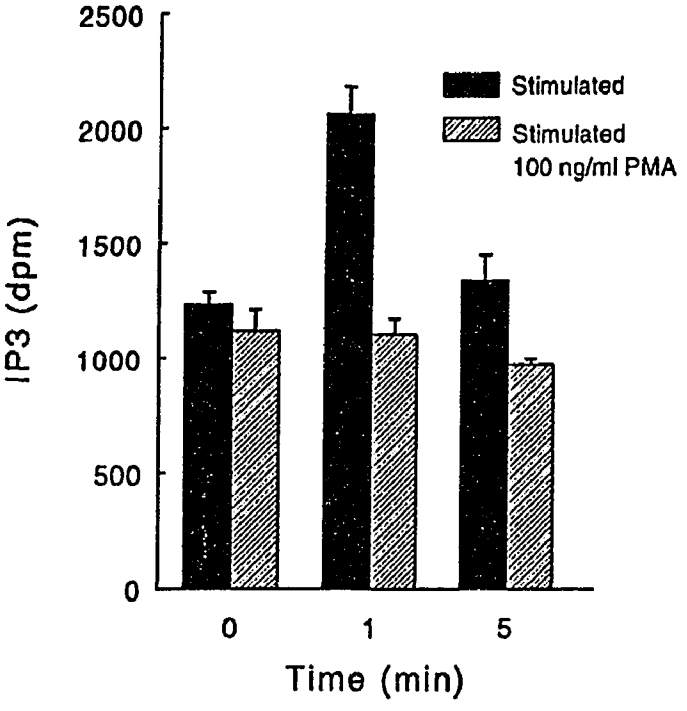


Figure 3-6. IP3 generation following crosslinking of FcγRIIA. PW16 cells were labeled for two days in medium containing [^{3}H]-myo-inositol. Labeled cells were preincubated either in the presence or in the absence of PMA for 15 min before stimulation by crosslinking bound IV.3 Fab with goat F(ab')$_2$ anti-mouse IgG. The cells were lysed in 10 mM formic acid, the extracted inositol phosphates were separated by an anion exchange chromatography, and IP3 quantified by a scintillation counter.



Chapter 4

Signaling mediated by FcγRIIA and FcγRIIIB in Frustrated phagocytosis

Summary

Binding of immune complexes to Fcγ receptors (FcγR) triggers a number of cellular responses, including phagocytosis. Using the murine macrophage cell line $P388D_1$ transfected with huFcγRIIA and human neutrophils, we examined the signaling events following the tethering of cell surface receptors to glass. Ligation of FcγRIIA (CD32) expressed in $P388D_1$ cells or neutrophils resulted in dramatic cell spreading, F-actin polymerization, and prolonged tyrosine phosphorylation, events that also occur during normal FcγR-mediated phagocytosis. The "frustrated phagocytosis" was inhibited by tyrosine kinase inhibitors and by the intracellular Ca^{2+} chelator BAPTA-AM, indicating a requirement for tyrosine phosphorylation and Ca^{2+} flux. Ligation to glass of the GPI-anchored neutrophil FcγRIIIB (CD16) resulted in a shape change of 5-10% of neutrophils. However, a series of protein kinase C inhibitors dramatically increased the percentage of neutrophils responding to FcγRIIIB ligation and to sub-optimal FcγRII ligation. Protein kinase C activation in neutrophils serves to blunt phagocytosis, particularly following FcγRIIIB activation, perhaps by inhibiting Ca^{2+} flux.

Introduction

Phagocytosis, tyrosine kinase activation, and Ca^{2+} flux, are major early events triggered by the activation of FcγRIIA. Compared to the antigen receptor activation motif (ARAM) found in other multichain immune recognition receptors, in which 10-11 residues separate the tyrosine resides in the dyad YXXL motifs (Keegan and Paul, 1992), FcγRIIA contains a modified ARAM with 15 residues separating the tyrosine residues. The events that differentiate phagocytosis of large particles from endocytosis of small complexes are not completely understood. We reported previously the correlations between tyrosine phosphorylation of Shc, PLC-γ1, and Syk and Ca^{2+} flux in $P388D_1$ cells expressing human FcγRIIA (Shen et al., 1994), in which immune complexes were endocytosed upon FcγRIIA crosslinking in a temperature-sensitive manner. However, we found dramatic differences in the requirements for immune complex endocytosis compared to phagocytosis of IgG-sensitized erythrocytes (Odin et al., 1991), which was inhibited by internal calcium chelators and did not occur in macrophages transfected with FcγRIIA mutants that endocytose complexes normally but fail to trigger a Ca^{2+} flux.

Although there is agreement that FcγRIIA plays an important role in signal transduction, there are contrasting reports on the functional capacity of the glycan phosphatidyl inositol (GPI)-anchored FcγRIIIB expressed on human neutrophils. Thus, although some groups have reported Ca^{2+} flux (Kimberly et al., 1990; Salmon

et al., 1991; Hundt et al., 1993; Naziruddin et al., 1992) and phagocytosis (Salmon et al., 1987) following FcγRIIIB engagement, others have negated any involvement of FcγRIIIB in triggering of the Ca^{2+} flux (Huizinga et al., 1990; Macintyre et al., 1989; Wirthmueller et al., 1992), the superoxide burst (Tosi and Berger, 1988; Huizinga et al., 1990), and ADCC (Graziano and Fanger, 1987; Selvaraj et al., 1989). Therefore, a careful study of signaling by neutrophil FcγRIIIB is warranted.

The role of protein kinase C (PKC) in phagocytic signaling is also a matter of controversy. PKC inhibitors have been reported to block the formation of focal adhesions triggered by β1 integrin interaction with fibronectin (Woods and Couchman, 1992). Phagocytosis by monocytes of 3 μm latex beads coated with IgG was associated with PKC translocation to membranes, and ingestion of latex was partially inhibited by calphostin C, albeit at very high concentration (Zheleznyak and Brown, 1992). In contrast to these results, others found that the kinase inhibitor staurosporine had little effect on neutrophil phagocytosis mediated by FcγR but strongly inhibited CR3-mediated phagocytosis (Roubey et al., 1991). There is also controversy about the role of $[Ca^{2+}]_i$ among the secondary events following activation of FcγR. An increase in $[Ca^{2+}]_i$ is one of the early events triggered by ligation of FcγR, but mouse macrophages and macrophage cell lines have been reported to phagocytose at very low $[Ca^{2+}]_i$ levels (Di Virgilio et al., 1988).

In the present study, we have examined the signaling events mediated via FcγRIIA and FcγRIIIB using a model for frustrated phagocytosis. Cells were plated

on a glass surface covalently coupled with either $F(ab')_2$ anti-mouse IgG or streptavidin and then the receptors were ligated to the glass by addition of anti-FcγR Fab or biotinylated Fab. We demonstrate signaling by both FcγRIIA and FcγRIIIB and describe an unanticipated role of protein kinase C in the down-regulation of the signaling triggered by ligation of GPI-anchored neutrophil FcγRIIIB.

Results and Discussion

Response to FcγR ligation of P388D$_1$ cells transfected with wt or mutated FcγRIIA

To facilitate the study of the phagocytic response of macrophages and neutrophils following ligation of FcγR, we coupled either F(ab')$_2$ anti-mouse IgG or streptavidin to glass activated with an aminosilane reagent and derivatized with glutaraldehyde (Werb et al., 1989), plated cells on the prepared substrate, and added either anti-FcγRII mAb IV.3 Fab or biotinylated IV.3 Fab. Unlike bare glass, the protein-coated glass did not activate neutrophil adhesion and spreading. The ligation to glass of human FcγRIIA stably expressed in P388D$_1$ cells (PW16) resulted in a dramatic spreading and flattening of the cells (Fig. 1B) compared to the control (Fig. 1A). The spreading was maximal after 10 min, and persisted for at least 4 hr. Equivalent results were obtained when cells were plated on glass coated with streptavidin and triggered with biotinylated-IV.3 Fab (not shown).

The shape change we observed was dependent on signaling mediated by the receptor, and not simply due to ligation of the receptor to glass, as shown by the failure of P388D$_1$ cells transfected with the FcγRIIA deletion mutants Δ264 (Fig. 1D) and Δ233 (Fig. 1F) to spread under the same conditions as cells transfected with wt FcγRIIA (Fig. 1B). These phenomena were quantified by computer analysis of the cell area (Fig. 2). The histograms show that > 85% of P388D$_1$ cells expressing wt

FcγRIIA had areas > 40 μm^2 10 min after FcγRIIA ligation, whereas > 85% of $P388D_1$ cells expressing the Δ233 and Δ264 deletion mutants had areas < 40 μm^2 at similar times (Fig. 2). The Δ233 mutation, which deletes both YXXL motifs in the cytoplasmic domain, is completely non-functional, mediating neither tyrosine phosphorylation, Ca^{2+} flux, internalization of small complexes, or phagocytosis. However, the Δ264 deletion of FcγRIIA, which removes the COOH-terminal YXXL motif, preserves normal endocytosis of small complexes, but abolishes phagocytosis of IgG-sensitized erythrocytes and Ca^{2+} flux (Odin et al., 1991).

To determine if the morphological change of PW16 cells we observed upon FcγRIIA ligation was dependent on Ca^{2+} flux, cells were loaded with the Ca^{2+} chelator BAPTA by incubation with its acetoxymethylester derivative (BAPTA-AM) (100 μM, 30 min, 37°C) before addition of IV.3 Fab. This treatment blocked the spreading of PW16 cells in response to IV.3 Fab (Fig. 1G), as well as the spreading of monocytes and neutrophils in response to FcγRII ligation (not shown). The requirement for elevated $[Ca^{2+}]_i$ in the phagocytic response mediated by FcγR is controversial, as some have reported it to be necessary (Lew et al., 1985) while others have described a normal response in inflammatory peritoneal macrophages buffered for $[Ca^{2+}]_i$ (Di Virgilio et al., 1988) and in neutrophils depleted of $[Ca^{2+}]_i$ (Della Bianca et al., 1990). In our phagocytosis model, in which cells are adherent to the substrate before ligation of receptors is initiated, a $[Ca^{2+}]_i$ transient appears to be necessary in macrophage cell lines, neutrophils, and monocytes.

Preincubation of cells with genestein (10 μg/ml, 30 min), a tyrosine kinase inhibitor, also blocked the shape change in response to FcγRIIA ligation (Fig. 1H), as did herbimycin A (10 μM, 16 hr) (not shown). As expected, the dramatic spreading of the cells in response to FcγRIIA ligation is a consequence of F-actin polymerization. Incubation of permeabilized cells with rhodamine-phalloidin (Fig. 1E) reveals strong F-actin staining, particularly in the pseudopodia at the margins of the cell.

Protein tyrosine phosphorylation

We reported previously that, in suspended cells, crosslinking of IV.3 Fab bound to FcγRIIA expressed on P388D$_1$ cells in suspension with F(ab')$_2$ anti-mouse IgG led to the rapid and transient tyrosine phosphorylation of a distinct set of proteins, including Shc and the tyrosine kinase Syk (Shen et al., 1994). We wanted to determine whether the rapid dephosphorylation observed in this system (Fig. 3A) is an obligatory desensitization event or is a consequence of the endocytosis of the complexes, which takes place at roughly the same rate as dephosphorylation. In contrast to the brief (5-10 min) duration of tyrosine phosphorylation in cells endocytosing small complexes (Fig. 3A), P388D$_1$ cells under conditions of frustrated phagocytosis showed persistent tyrosine phosphorylation, lasting for hours (Fig. 3B). Shc and Syk were prominently phosphorylated following activation by frustrated

phagocytosis (Fig. 3C), and their phosphorylation was maintained for hours. These results suggest that endocytosis of the complexes results in kinase inactivation and/or protein phosphotyrosine phosphatase activation. Under conditions where the complexes cannot be internalized, the final extent of tyrosine phosphorylation reflects a balance between tyrosine kinase and protein phosphotyrosine phosphatase activities.

Human neutrophil response to FcγR ligation

Human neutrophils express products of two FcγR genes, FcγRIIA, a class I transmembrane glycoprotein, and FcγRIIIB, which is anchored to the membrane by a GPI moiety. Ligation of neutrophil FcγRIIA resulted in dramatic spreading (Fig. 4A) compared to the unstimulated control (Fig. 4B). As observed previously for macrophages, the neutrophil response to FcγRIIA ligation was inhibited by loading cells with BAPTA (Fig. 4C). In contrast to FcγRIIA, ligation of FcγRIIIB resulted in a modest response, with only about 10% of cells showing a change in morphology (Fig. 4D). This was, however, consistently observed following FcγRIIIB ligation with cells placed on both the streptavidin- (not shown) and $F(ab')_2$ goat anti-mouse IgG-coated glass.

Since phorbol myristate acetate (PMA) also triggers neutrophil spreading, we initially hypothesized that activation of the GPI-anchored FcγRIIIB might lead to sub-optimal activation of protein kinase C (PKC) and a reduced percentage of responding

cells. However, pre-treatment of neutrophils with the PKC inhibitor calphostin C (1.2 μM, 30 min), which by itself had no effect on morphology (Fig. 4E), dramatically increased the percentage of cells responding to FcγRIIIB ligation (Fig. 4F). This observation was repeated with the inhibitors H7 (20 μM), H8 (50 μM), and staurosporine (100 nM), although the staurosporine treatment resulted in abnormal neutrophil morphology (not shown). A concentration of 1.2 μM calphostin C resulted in optimal spreading, with a lower effect at 0.6 μM (Fig. 5A). The effect of PKC inhibitors on the stimulation of neutrophil shape changes was not limited to the GPI-anchored FcγRIIIB. When the amount of IV.3 Fab was decreased to levels resulting in sub-optimal stimulation of FcγRIIA, calphostin C treatment had a similar enhancing effect on neutrophil spreading (Fig. 5A).

The effect of calphostin C on P388D$_1$ cells transfected with FcγRIIA was also examined (Fig. 5B). In contrast to the stimulatory effect on neutrophils, calphostin C had a slight inhibitory effect on FcγRIIA-induced shape change of P388D$_1$ cells (Fig. 5). This is in agreement with the observation that calphostin C, at the concentrations used here, had at most a modest inhibitory effect on monocyte phagocytosis of IgG-coated latex beads, and only much higher calphostin C concentrations lead to significant inhibition of phagocytosis (Zheleznyak and Brown, 1992).

Interpretation of these results is complicated by the multiplicity of protein kinase C isozymes, at least 12 known at present (Dekker and Parker, 1994). The PKC-δ isoform is highly expressed in myeloid cells (Mischak et al., 1993) and is

tyrosine- phosphorylated upon activation with PMA (Li et al., 1994). Indeed, following incubation of $P388D_1$ cells with PMA, tyrosine phosphorylation of an 74,000 Mr protein (the size of PKC-δ) was induced (Fig. 6). Furthermore, immunoblotting of an anti-phophotyrosine immunoprecipitate with an anti-PKC-δ antibody revealed the presence of PKC-δ in both $P388D_1$ cells stimulated by FcγRIIA ligation, and treated with PMA (Fig. 6). It is possible that other PKC isoforms are also activated by FcγRIIA crosslinking. Thus it is likely that PMA acts as a more general agonist than stimulation via FcγR crosslinking. Furthermore, it is likely that PKC isozymes are differentially sensitive to PKC inhibitors. For example, PMA-induced actin assembly and cell ruffling are not inhibited by calphostin C at concentrations that inhibit superoxide production (Downey et al., 1992). Calphostin C (1.2 μM) had no effect on PMA-induced spreading of neutrophils or macrophages (not shown). In addition, calphostin C, by itself, is reported to stimulate the production of platelet activating factor and arachidonic acid, and to induce neutrophil homotypic aggregation (Svetlov and Nigam, 1993). In our study, we have not observed calphostin C-induced shape changes without FcγR ligation. The stimulation of frustrated phagocytosis we observe could, however, result from the synergistic effect of two sub-threshold stimuli. Since we have observed previously a requirement for $[Ca^{2+}]_i$ in phagocytosis, we next investigated the effect of calphostin C on FcγR-triggered $[Ca^{2+}]_i$ flux.

$[Ca^{2+}]_i$ changes ***(done by Frank Y.S. Chuang, a MSTP student in the lab)***

Although only 10-15% of neutrophils activated by ligating FcγRIIIB to glass show shape changes, the GPI-anchored receptor can trigger a $[Ca^{2+}]_i$ transient that is similar in magnitude to that elicited by crosslinking FcγRIIA or adding the chemotactic peptide fMetLeuPhe (FMLP). Crosslinking FcγRIIIB on neutrophils in suspension resulted in an optimal signal approaching 1 μM $[Ca^{2+}]_i$ (Fig. 7). The magnitude of the signal was not appreciably diminished by the presence of EGTA in the bathing medium, confirming that the Ca^{2+} was released from internal stores (not shown). Furthermore, ratio imaging of indo-1 loaded neutrophils triggered by ligation of FcγRIIIB showed that essentially all cells respond with elevated $[Ca^{2+}]_i$ (not shown). At all concentrations of 3G8 Fab tested, calphostin C pretreatment of neutrophils led to an approximate two-fold increase in the magnitude of $[Ca^{2+}]_i$ flux (not shown). A similar potentiation of $[Ca^{2+}]_i$ flux by calphostin C treatment was seen after crosslinking of FcγRIIA as well as after stimulation with formyl-Met-Leu-Phe (fMLP). We often observed a slight increase in the baseline $[Ca^{2+}]_i$ following treatment with calphostin C. Moreover, in cells pretreated with calphostin C, we consistently observed a prolonged increase of $[Ca^{2+}]_i$ following the fast transient elicited by both FcγR and chemotactic peptide receptor stimulation. These results agree with previous work (Svetlov and Nigam, 1993), and suggest that calphostin C may interfere with the re-uptake of Ca^{2+} into internal compartments, or may prolong the opening of secondary Ca^{2+} channels leading to flux across the plasma membrane

(Krause et al., 1993). The stimulation in frustrated phagocytosis mediated by FcγR ligation may be a consequence of the elevated $[Ca^{2+}]_i$ transient, and suggests that the PKC isozyme stimulated by FcγR ligation inhibits the activity of PLC-γ2, the isozyme reported to be activated in neutrophils (Dusi et al., 1994), or modulates the release of Ca^{2+} by the IP3 receptor (Ferris et al., 1991).

β-2 Integrin role in frustrated phagocytosis

When erythrocytes are coated with both C3bi and IgG, they are phagocytosed much more avidly than when coated with either ligand alone, suggesting that receptors for IgG and C' act in synergy (Ehlenberger and Nussenzweig, 1977). The avidity of ligand binding by β-2 integrins is increased rapidly by stimulation of neutrophils and monocytes with either PMA or chemotactic factors (Wright and Meyer, 1986). MAbs that recognize activation epitopes on Mac-1 (CD11b/CD18) and block ligand binding have been defined (Diamond et al., 1993), and inhibition of neutrophil adhesion to IgG coated plates by anti-CD18 mAbs has also been reported (Kusunoki et al., 1994). However, the mAbs in the latter study were tested as intact IgG, which might interfere with FcγR binding. To test the hypothesis that β-2 integrin activation might play a key role in phagocytosis, in addition to the "zipper" effect of FcγR's interacting with their ligands, we tested the effect of $F(ab')_2$ fragments of the inhibitory anti-CD18 mAb 1B4 on frustrated phagocytosis. The glass surface was

coated with streptavidin, and neutrophils were triggered with biotinylated IV.3 Fab, which should not interfere with the binding of the 1B4 reagent. As seen in Fig. 7C, the spreading of the neutrophils in response to FcγRIIA ligation (Fig. 7B) was inhibited dramatically by blockade of the β-2 integrins. The $F(ab')_2$ mAb 1B4 had no detectable heavy chain that could interfere with FcγR ligation (Fig. 7E), nor was there any inhibition of binding of biotinylated IV.3 to the neutrophils by prior incubation with the 1B4 $F(ab')_2$ (Fig. 7D).

Our results suggest that the shape changes observed upon ligation of FcγRIIA and FcγRIIIB reflect the final step in a multi-component process, beginning with the activation of the tyrosine kinase cascade followed by the activation of PLC-γ1 (in macrophages) or PLC-γ2 (in neutrophils). The increase in $[Ca^{2+}]_i$, due to the release of internal Ca^{2+} stores, is then required for the activation of the β-2 integrin receptors and conversion of G-actin to F-actin. The mechanism of activation of the β-2 integrin receptors is not known, although the production of an unsaturated fatty acid or isoprenoid compound is suggested to modulate the increase in affinity associated with activation (Hermanowski Vosatka et al., 1992).

It is clear that FcγRIIA, is a much more potent trigger of the phagocytic response than is the GPI-anchored FcγRIIIB. However, ligation of neutrophil FcγRIIA and FcγRIIIB probably activates some of the same signaling pathways. In both cases the peak $[Ca^{2+}]_i$ was elevated by calphostin C pretreatment of the neutrophils, presumably by regulation of the activity of PLC-γ2. In contrast, the peak $[Ca^{2+}]_i$

resulting from FMLP stimulation, which activates PLC-β via a heterotrimeric G_q protein, was unaffected by calphostin C. It is likely that there is interaction between FcγRIIA and FcγRIIIB, as has been suggested by several investigators (Boros et al., 1991; Salmon et al., 1991; Naziruddin et al., 1992). Blockade of FcγRIIA or FcγRIIIB by mAbs inhibits mobilization of internal Ca^{2+} triggered by immune complexes (Brunkhorst et al., 1992) and blockade of FcγRIIA inhibits degranulation triggered by IgM anti-FcγRIII mAbs (Boros et al., 1991). The GPI-anchored FcγRIIIB may require FcγRIIA for signaling in the same way as GPI-anchored molecules expressed by T cells require the TCR/CD3 complex to mediate signal transduction (Bamezai et al., 1988; Gunter et al., 1987).

Acknowledgments

We would like to thank Rui Da Costa and Heikki Väänänen for help in the image based analysis of cell areas. This work was supported by PHS grants AI-24322 and AI-24671.

Figure 4-1. Response to FcγR ligation of P388D$_1$ cells transfected with wt or mutated FcγRIIA. Cells were plated on glass coverslips coated with F(ab')$_2$, and activated by adding

1 μg/ml anti-γRIIA mAb IV.3 Fab. **A.** wt FcγRIIA, control; **B.** wt FcγRIIA, stimulated; **C.** Δ264, control; **D.** Δ264, stimulated; **E.** Δ233, control; **F.** Δ233, stimulated; **G.** wt FcγRIIA preincubated with 100 μM BAPTA-AM for 30 min, then stimulated; **H.** wt FcγRIIA preincubated with 10 ng/ml genestin for 30 min, then stimulated; **I.** wt FcγRIIA control cells were permeabilized with 0.1% Triton X-100, and stained with 0.2 mg/ml rhodamine-phalloidin; **J.** wt FcγRIIA stimulated cells were permeabilized with 0.1% Triton X-100, and stained with 0.2 mg/ml rhodamine-phalloidin.

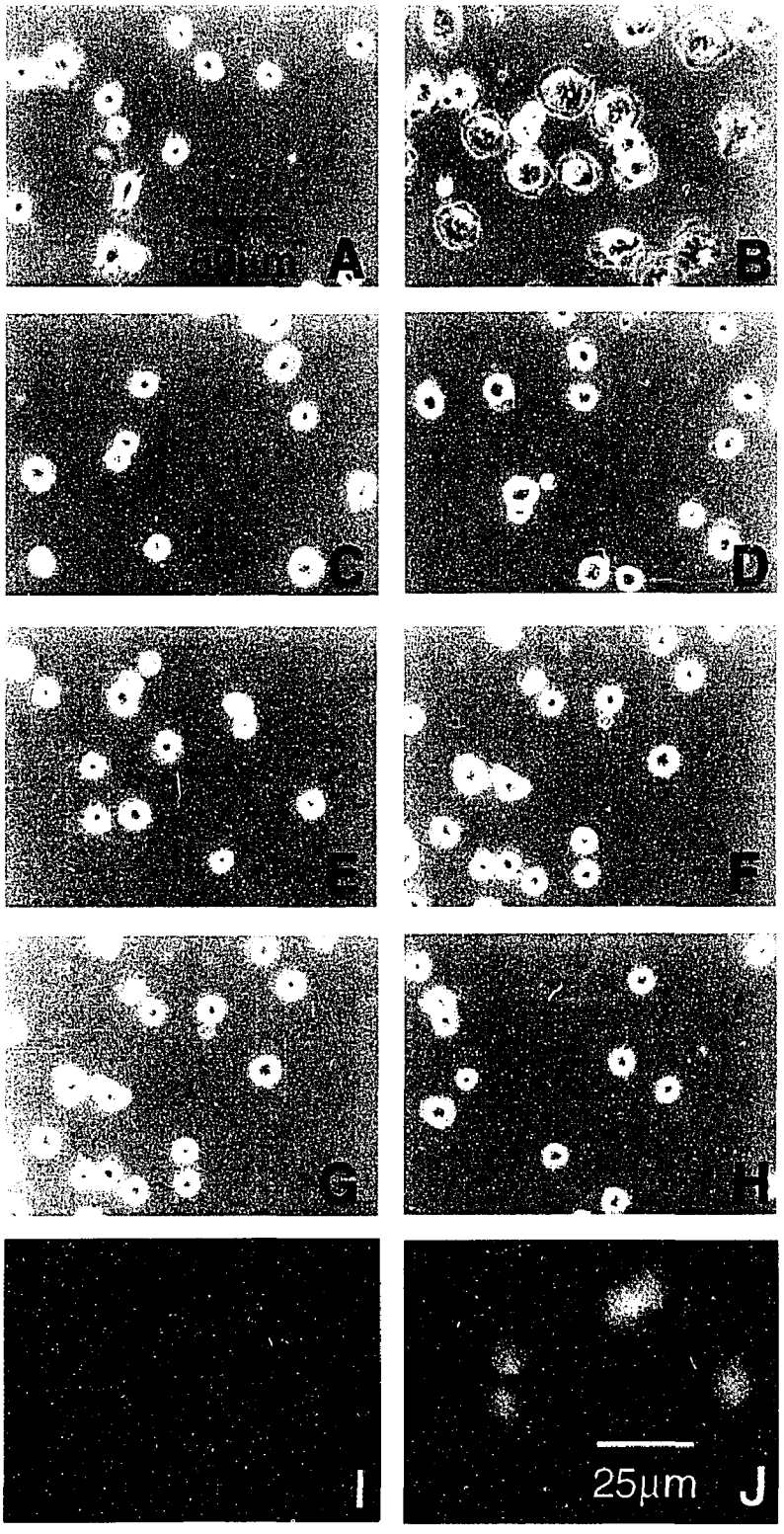

A
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Figure 4-2. Histogram plots of surface areas of cells expressing wt and mutated FcγRIIA during frustrated phagocytosis. Surface areas of control and activated cells were quantified by computer analysis as described in Experimental Procedures.

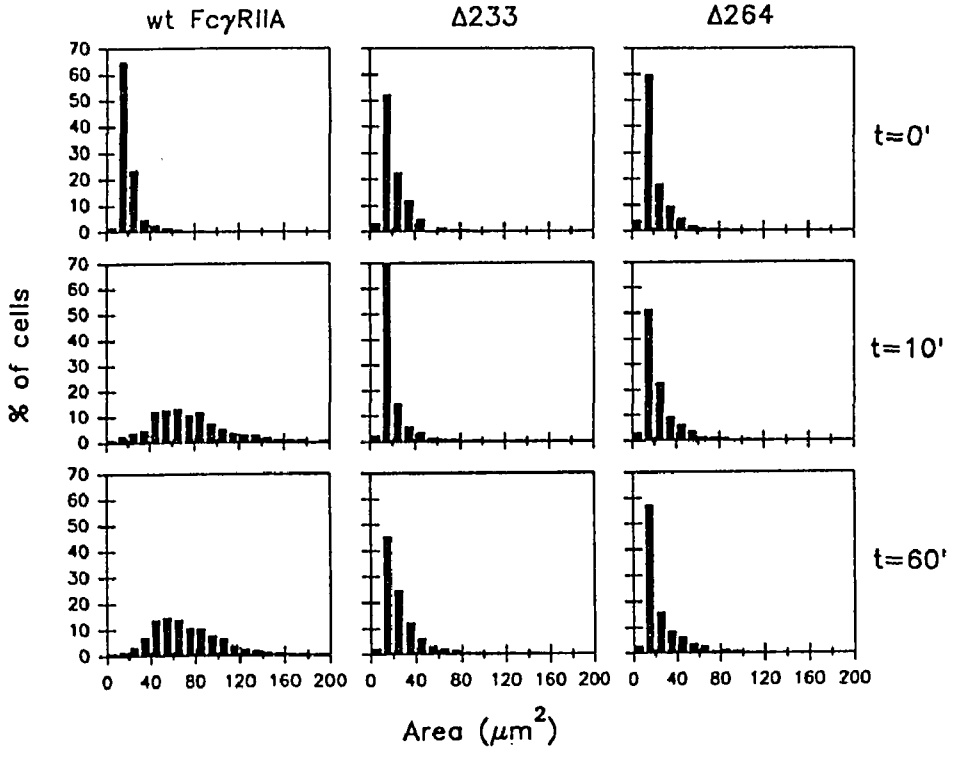


Figure 4-3. Protein tyrosine phosphorylation during frustrated phagocytosis. FcγRIIA expressed in P388D$_1$ cells was crosslinked, and at intervals the cells were lysed in 0.5% NP-40 lysis buffer. After SDS-PAGE, proteins were transferred to nitrocellulose membranes, and blotted with anti-phosphotyrosine mAb 4G10. **A.** Cells in suspension **B.** Receptors immobilized on glass; **C.** Immunoprecipitation of Shc and Syk from cells during frustrated phagocytosis.

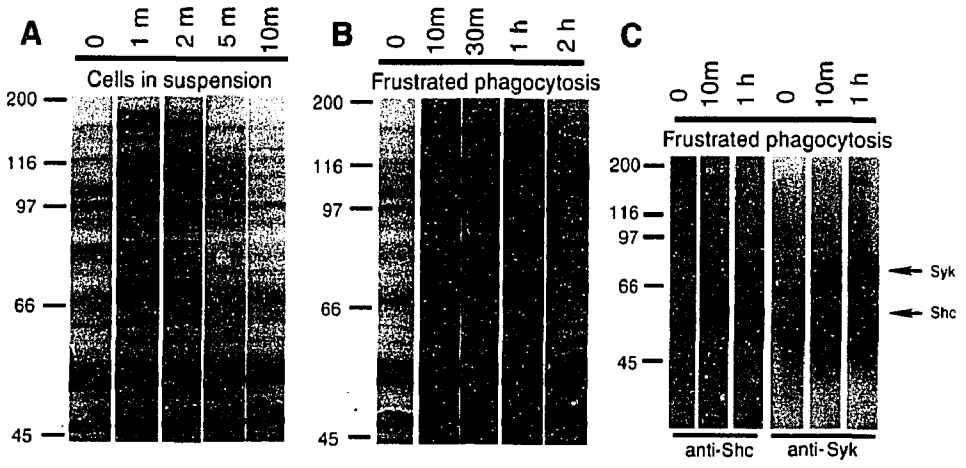


Figure 4-4. Human neutrophil response to FcγR ligation. Neutrophils purified from human blood by centrifugation on a histopaque gradient were plated on $F(ab')_2$ anti-mouse IgG coated coverslips, and triggered with either 1 μg/ml anti-FcγRIIA mAb IV.3 Fab or 5 μg/ml anti-FcγRIIIB mAb 3G8 Fab. **A.** Cells activated by IV.3 Fab; **B.** non-stimulated cells; **C.** Cells preincubated with 100 μM BAPTA-AM for 30 min, then activated with IV.3 Fab; **D.** Cells activated with 3G8 Fab; **E.** Cells preincubated with 10 ng/ml genestin for 30 min, then activated with IV.3 Fab; **F.** Cells preincubated with 1.2μM calphostin C for 30 min, then activated with 3G8 Fab.

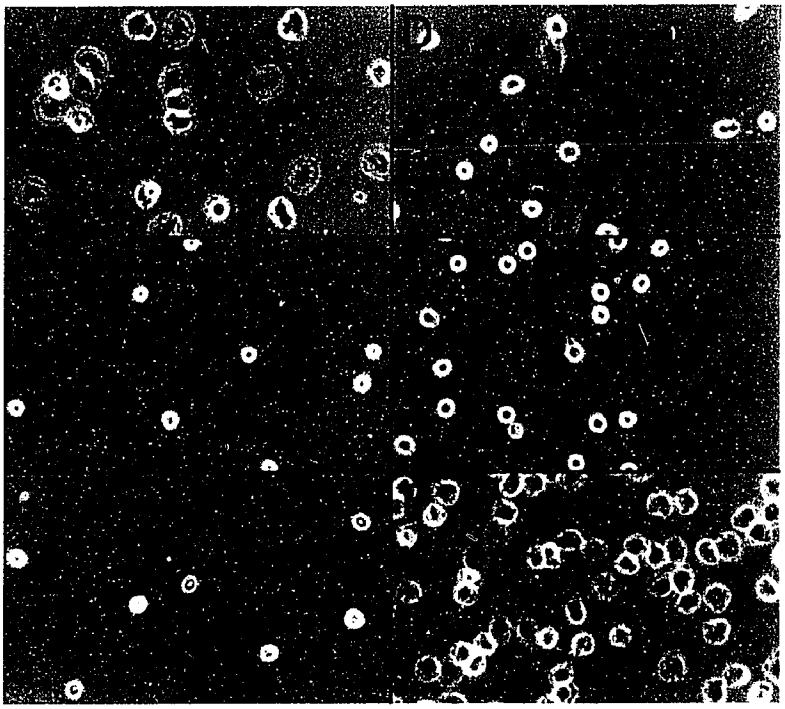


Figure 4-5. Effects of calphostin C on cell spreading in both human neutrophils (**A**) and mouse macrophages (**B**). Cells were treated (37°C, 30 min) with calphostin C, and then cross-linked with either IV.3 Fab (1 μg/ml, optimal and 0.1 μg/ml, sub-optimal) or 5 μg/ml 3G8 Fab. The percentage of spread cells was quantified.

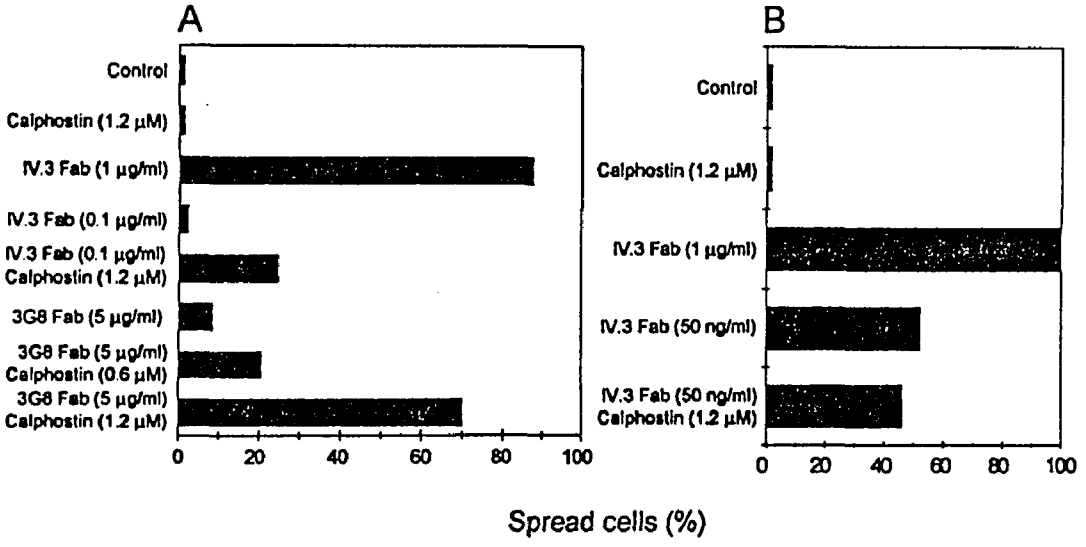


Figure 4-6. Tyrosine phosphorylation of PKC-δ. P388D$_1$ cells transfected with FcγRIIA were plated on goat anti-mouse IgG F(ab')$_2$ coated glass dishes in either presence or absence of 100 ng/ml PMA, and the receptors were then activated by adding IV.3 Fab (1 μg/ml) for 30 min at 37^{0}C. Cells were then scraped and lysed in 0.5% NP-40 lysis buffer. The cell lysates were blotted with mAb 4G10, and the 4G10 immunoprecipitates were probed with a polyclonal anti-PKC-δ IgG.

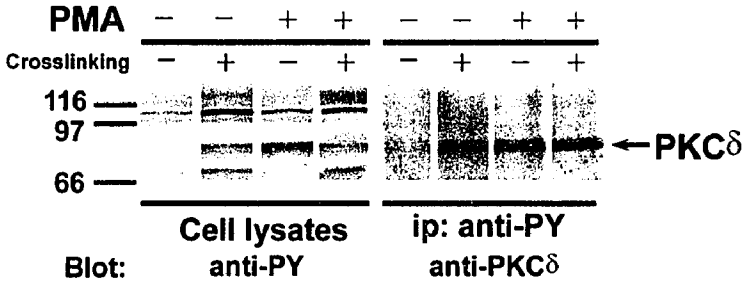
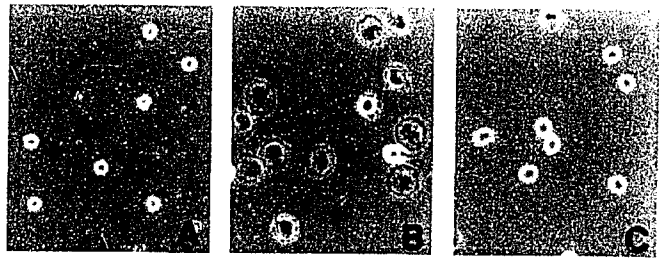
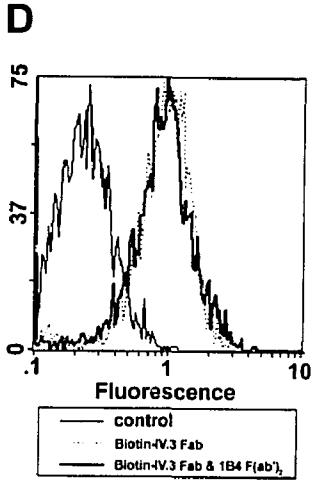
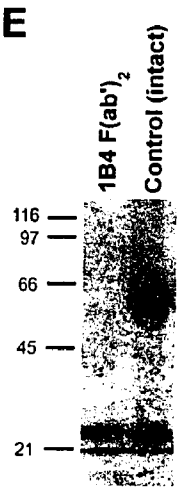


Figure 4-7. The effect of anti-β2 integrin mAb 1B4 F(ab')$_2$ on frustrated phagocytosis. Neutrophils were plated on streptavidin coated coverslips in either absence or presence of 1 μg/ml 1B4 F(ab')$_2$ at 37°C for 30 min, then activated by adding 1 μg/ml biotinylated anti-FcγRIIA mAb IV.3 Fab. **A.** non-stimulated cells; **B.** stimulated cells; **C.** stimulated cells in the presence of 1 μg/ml 1B4 F(ab')$_2$. **D.** Neutrophils were incubated with 1 μg/ml biotinylated IV.3 Fab in either presence (solid line) or absence (dotted line) of 1 μg/ml 1B4 F(ab')$_2$ at 37°C for 30 min, then stained with streptavidin-FITC for 45 min at 4°C, and assayed by FACS. **E.** 1B4 F(ab')$_2$ and control intact mAb were run on reducing SDS-PAGE, and visualized by Coomassie Blue staining.







Chapter 5

Roles of Phosphatidylinositol 3-kinase in FcγRIIA and FcγRIIIB-Mediated phagocytosis and $[Ca^{2+}]_i$ Transient

Summary

The PI 3-kinase inhibitor, wortmannin, inhibited (Ki = 2 x 10^{-9} M) frustrated phagocytosis triggered by human FcγRIIA in a transfected P388D_1 murine macrophage line and in human neutrophils. Neutrophil frustrated phagocytosis mediated by glycan phosphatidyl inositol (GPI) anchored neutrophil FcγRIIIB was similarly inhibited. Wortmannin had no effect on endocytosis of cell surface complexes of human FcγRIIA, gross tyrosine phosphorylation patterns or the tyrosine phosphorylation of macrophage PLC-γ1 in response to FcγRIIA ligation. However, the release of inositol trisphosphate (IP3) was totally inhibited by 10 nM wortmannin. Both neutrophil FcγRIIA- and FcγRIIIB-stimulated release of $[Ca^{2+}]_i$ from internal stores were inhibited by wortmannin with Ki = 2-2.6 x 10^{-9} M. Confirming other reports, we found FcγR stimulation resulted in stimulation of PI 3-kinase activity. We found that enhanced PI 3-kinase activity was co-immunoprecipitated with PLC-γ1. Only a small amount of p85 was found to associate with PLC-γ1, we cannot detect the tyrosine phosphorylated p85 regulatory subunit of PI 3-kinase in the immunoprecipitate of PLC-γ1, although tyrosine phosphorylated p85 is readily visible in the p85 immunoprecipitate. We suggest that stimulated PI 3-kinase activity is complexed with PLC-γ1 and that tyrosine phosphorylation of PLC-γ1, while perhaps necessary for stimulated activity of the enzyme and release of IP3, is not sufficient -- that activity of PLC-γ1 is dependent on PI 3-kinase activity.

Introduction

Fc Receptors for IgG, FcγRs, mediate a number of physiological functions including calcium mobilization, endocytosis, phagocytosis and activation of protein kinase. We reported previously that in P388D$_1$ cells, a murine macrophage cell line, crosslinking of transfected human FcγRIIA led to $[Ca^{2+}]_i$ flux, receptor endocytosis and phagocytosis of opsonized erythrocytes (Odin et al., 1991). However, a deletion mutant of human FcγRIIA (Δ264) lacking the 17 carboxyl-terminal residues was able to mediate endocytosis but failed to mediate $[Ca^{2+}]_i$ flux and phagocytosis, suggesting that there are different signaling requirements for these events.

PI 3-kinase phosphorylates PI, PI 4-P, PI 4,5-P_2 on the D-3 position of the inositol ring. The PI 3-kinase products may be a new class of second messager, the physiological roles of which are not understood yet. Alternatively, the relevant substrates for PI 3-kinase may be the proteins instead of lipids, as is suggested in studies of PI 3-kinase-mediated serine phosphorylation of IRS1 following insulin stimulation (Lam et al., 1994). Purified PI 3-kinase is a heterodimer consisting of 85 Kd (p85) regulatory subunit and 110 Kd (p110) catalytic subunit. The p85 subunit of PI 3-kinase contains SH2 and SH3 domains, but no catalytic sequence (Escobedo et al., 1991; Otsu et al., 1991; Skolnik et al., 1991), the p110 subunit contains the catalytic domain (Hiles et al., 1992). The SH2 domains of p85 direct the protein interaction of PI 3-kinase and phosphotyrosine-containing proteins (McGlade et al., 1992; Klippel et al., 1992) with the Y-X-X-M motif (Fantl et al., 1992; Kazlauskas et

al., 1992). The pivotal role of PI 3-kinase in cell signaling in a variety of systems is beginning to emerge (Cantley et al., 1991). FcγRIIIA stimulation leads to activation of PI 3-kinase in NK cells (Kanakaraj et al., 1994). Enhanced PI 3-kinase activity is associated with both activated receptor protein tyrosine kinase such as EGFR, PDGFR, and non-receptor protein tyrosine kinase, such as Src and Fyn (Cantley et al., 1991; Koch et al., 1991; Fukui et al., 1991b). However, there was always PI 3-kinase activity detected in resting states. The exact roles of PI 3-kinase is not fully understood. *VPS*34 gene product, a yeast p110 homologue with PI 3-kinase activity, is essential for protein sorting in yeast, suggesting a possible role of PI 3-kinase in mammalian protein sorting (Schu et al., 1993). Recently, PI 3-kinase was shown to be a downstream target of Ras (Rodriguez Viciana et al., 1994), and mediated PDGF- and insulin-dependent $pp70^{S6K}$ activation (Chung et al., 1994).

Wortmannin, a fungal metabolite, is a selective and potent inhibitor of PI 3-kinase (Okada et al., 1994b). The critical role of PI 3-kinase thus can be demonstrated using wortmannin in nanomolar concentrations. It was shown that wortmannin blocked fMLP-induced neutrophil stimulation (Okada et al., 1994b) and inhibited insulin-induced glucose transport and antilipolysis in rat adipocytes (Okada et al., 1994a) as a result of inhibition of PI 3-kinase. More recently, wortmannin was demonstrated to inhibit FcγR-mediated phagocytosis in U937 cells or guinea pig neutrophils, and PI 3-kinase activity was detected in anti-phosphotyrosine immunoprecipitates (Ninomiya et al., 1994).

In the present study, we have examined the role of PI 3-kinase in FcγRIIA and FcγRIIIB signaling in both macrophages and neutrophils. We demonstrate that wortmannin inhibited FcγRIIA- and FcγRIIIB-mediated frustrated phagocytosis, IP3 production and $[Ca^{2+}]_i$ flux but not endocytosis as a result of inhibiting PI 3-kinase specifically. Increased PI 3-kinase activity was found to associate with activated PLC-γ1 in $P388D_1$ macrophage cells.

Results

Wortmannin inhibited FcγRIIA and FcγRIIIB-mediated frustrated phagocytosis

We used a model of frustrated phagocytosis to analyze the role of PI 3-kinase in FcγR-mediated phagocytosis. Goat anti-mouse $F(ab')_2$ IgG was chemically coupled to glass coverslips. After plating either $P388D_1$ cells expressing human FcγRIIA, or human neutrophils expressing both FcγRIIA and FcγRIIIB on the coated coverslips, the cells were triggered with either mAb IV.3 Fab (anti-FcγRIIA) or mAb 3G8 Fab (anti-FcγRIIIB). The ligation to glass of human FcγRIIA in both macrophages (Fig. 1B) and neutrophils (Fig. 1E) resulted in dramatic cell spreading and flattening of the cells (Fig. 1B) compared to control cells (Fig. 1A, D). Ligation to glass of human FcγRIIIB resulted in 10-20% cell spreading (Fig. 1F). Wortmannin, a highly cell permeable fungal metabolite, is a potent and selective inhibitor of PI 3-kinase (Okada et al., 1994b). Pretreatment of $P388D_1$ cells expressing human FcγRIIA with 10 nM wortmannin resulted in complete inhibition of cell spreading (Fig. 1C). Similarly, wortmannin inhibited the morphological changes of human neutrophils by crosslinking of either FcγRIIA (Fig. 1G) or GPI-anchored FcγRIIIB (Fig. 1H). Our data confirmed a previous report showing that wortmannin at 1 μM inhibited phagocytosis in guinea pig neutrophils (Ninomiya et al., 1994). However, we found inhibition of lower concentration of wortmannin, 10 nM instead of 1 μM.

Wortmannin inhibited $[Ca^{2+}]_i$ flux upon crosslinking of human FcγRIIA and human FcγRIIIB *(work done by Frank Y.S. Chuang, a MSTP student in the lab)*

We found previously that $[Ca^{2+}]_i$ flux is necessary for FcγR-mediated phagocytosis (Shen and Unkeless, unpublished result). To determine whether the inhibition of cell shape changes by wortmannin is due to the inhibition of $[Ca^{2+}]_i$ flux, we measured $[Ca^{2+}]_i$ flux after crosslinking of either FcγRIIA or FcγRIIIB in both macrophages and human neutrophils. Wortmannin inhibited $[Ca^{2+}]_i$ transient from internal stores with Ki = 2-2.6 x 10^{-9} M. The effect of wortmannin was specific for $[Ca^{2+}]_i$ transient due to FcγR stimulation, there was no effect of wortmannin on $[Ca^{2+}]_i$ transient resulting from chemotactic receptor ligation with fMLP.

Protein tyrosine phosphorylation of PLC-γ1 and p85 regulatory subunit of PI3K

PLC-γ1 cleaves phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate, leading to protein kinase C activation and the release of intracellular stores of Ca^{2+}, respectively. The event of tyrosine phosphorylation of PLC-γ1 is correlated with its activity in growth factor receptor signaling(Nishibe et al., 1990b; Morrison et al., 1990). Since wortmannin inhibited $[Ca^{2+}]_i$ flux triggered by FcγRIIA and FcγRIIIB crosslinking, we examined the effect of wortmannin on tyrosine phosphorylation of PLC-γ1 in $P388D_1$ macrophage cells. Wortmannin had no

obvious effect on overall tyrosine phosphorylation pattern (Fig. 2A). NP-40 detergent lysates of cells treated with or without various concentration of wortmannin were immunoprecipitated with anti-PLCγ1 IgG, and immunoblotted with antiphosphotyrosine mAb 4G10. Crosslinking of FcγRIIA resulted in PLC-γ1 tyrosine phosphorylation, which was not affected by wortmannin (Fig. 2B). We were unable to detect tyrosine phosphorylation of PLC-γ1 in human neutrophils. It was reported that in human neutrophils, PLC-γ2 was phosphorylated on tyrosine residues, and probably was a major player for the generation of IP3 (Dusi et al., 1994).

Tyrosine phosphorylation of p85 regulatory subunit of PI3K was also examined. We found p85 subunit of the macrophages was phosphorylated on tyrosine even under resting conditions, and that crosslinking of FcγRIIA did not significantly enhance the tyrosine phosphorylation of p85 subunit (Fig. 2C). As reported for PLC-γ1, wortmannin had no effect on tyrosine phosphorylation of the p85 subunit.

IP3 production was inhibited by wortmannin

Since wortmannin inhibited $[Ca^{2+}]_i$ flux upon crosslinking of FcγRIIA without affecting tyrosine phosphorylation of PLC-γ1 in P388D$_1$ cells, we next assayed IP3 production. Cells were labelled with [^{3}H] *myo*-inositol, and crosslinked with and without preincubation with wortmannin. Activation of FcγRIIA by crosslinking resulted in elevated IP3 levels, which were not seen in cells pretreated with 10 nM

wortmannin (Fig. 3). This result indicated that the activity of PLC-γ1 was regulated by PI 3-kinase, and strongly implies that IP3 released by PLC-γ1 was responsible for the $[Ca^{2+}]_i$ flux. Wortmannin, which had no effect on tyrosine phosphorylation of PLC-γ1, inhibited PLC-γ1 activity.

Association of PI 3-kinase activity in PLC-γ1 immunoprecipitates

The observation that wortmannin, a specific inhibitor for PI 3-kinase, inhibits PLC-γ1, suggests that there may be interaction between PI 3-kinase and PLC-γ1. PI 3-kinase has recently been shown to contain intrinsic serine kinase activity toward the IRS-1 insulin receptor substrate (Dhand et al., 1994; Lam et al., 1994), raising the possibility that PI 3-kinase regulates PLC-γ1 activity through serine phosphorylation. To determine if PI 3-kinase forms a complex with PLC-γ1, $P388D_1$ cell lysates before and after crosslinking were immunoprecipitated with a rabbit polyclonal anti-PLC-γ1 IgG followed by assay for PI kinase. No PI kinase activity was detected in normal rabbit IgG immunoprecipitates. However, in PLCγ-1 immuprecipitates, PI 3-kinase activity was detected. There is basal level PI 3-kinase activity associated with PLC-γ1 immunoprecipitates in control. PI 3-kinase activity was elevated 2-3 fold upon crosslinking FcγRIIA (Fig. 4). Cells preincubated with 10 nM wortmannin showed only basal level PI3K activity even after crosslinking FcγRIIA, indicating that elevated PI kinase activity is due to enhancement of PI 3-kinase (Fig. 4). As a

positive control, parallel cell lysates were immunoprecipitated with polyclonal serum directed against the p85 regulatory subunit of PI 3-kinase. The p85 immunoprecipitates had much more kinase activity than was found in the PLC-γ1 immunoprecipitates, but there was no stimulation of PI 3-kinase activity seen in the p85 immunoprecipitates from stimulated cells compared to control cells. The PI 3-kinase activity in the PLC-γ1 immunoprecipitates was inhibited 50% by 10 nM wortmannin. In the PI kinase assays of PLC-γ1 immunoprecipitates, we always observed a phosphorylated lipid species migrating with the mobility of phosphatidic acid. This labelled lipid was not seen in the p85 immunoprecipitates. Crosslinking of FcγRIIA led to enhanced production of phosphatidic acid, and wortmannin (10 nM) inhibited its formation to background level (Fig. 4). The above data suggested that diacylglycerol kinase was also associated with the complex. To confirm this observation, DAG was used instead of phosphatidylinositol as a substrate for the kinase assay. DAG was rapidly converted to phosphatidic acid by diacylglycerol kinase in PLC-γ1 immunoprecipitates (data not shown). The substrate for diacylglycerol kinase in PLC-γ1 immunoprecipitates is presumably due to cleavage of phosphatidyl inositol by PLC-γ1, and the newly generated DAG was rapidly re-phosphorylated to produce phosphatidic acid. Thus, the generation of phosphatidic acid also reflected PLC-γ1 activity. Triton X-100 was reported to inhibit PI 3-kinase activity while enhancing PI 4-kinase (Fukui et al., 1989), we found that inhibition of PI 3-kinase activity by Triton X-100 led no production of phosphatidic acid (data not

shown), suggesting that PI 3-kinase activity is necessary for PLC-γ1 activity. Alternatively, Triton X-100 may inhibit DAG kinase.

Discussion

FcγRIIA has a modified ARAM (antigen receptor activation motif), with greater spacing between the two Y-X-X-L motifs than the ARAM found in other multichain immune recognition receptors, such as T cell antigen receptor and B cell antigen receptor (Keegan and Paul, 1992). Crosslinking of transfected human FcγRIIA in $P388D_1$ macrophage cells led to receptor internalization, phagocytosis of IV.3-sensitized erythrocytes (Odin et al., 1991), and rapid tyrosine phosphorylation of cellular substrates including Shc, PLC-γ1, and a tyrosine kinase Syk (Shen et al., 1994). Ingestion of immune complexes versus erythrocytes clearly requires different intracellular events than endocytosis. For example, Δ264, a FcγRIIA mutant lacking the C-terminal 17 amino acid residues does not mediate phagocytosis or $[Ca^{2+}]_i$ transients upon FcγRIIA crosslinking, yet this mutant is able to endocytose the receptor to the same extent as wild type FcγRIIA (Odin et al., 1991).

We are interested in dissecting the requirements for FcγRIIA-mediated phagocytosis. We found that protein tyrosine phosphorylation and $[Ca^{2+}]_i$ flux were necessary for phagocytosis (Shen and Unkeless, unpublished results). With the availability of wortmannin, a fungal metabolite that specifically inhibits PI 3-kinase, we were able to examine the role of PI 3-kinase in this process. Wortmannin, even at high concentration, did not interfere with the receptor-mediated endocytosis of small complexes. In contrast, we showed that PI 3-kinase is clearly involved in FcγRIIA-mediated frustrated phagocytosis as demonstrated by the morphologic changes of both

macrophages and neutrophils.

We were surprised to find that inhibition of PI 3-kinase activity by wortmannin resulted in inhibition of $[Ca^{2+}]_i$ flux and IP3 production, implicating that the activity of PLC-γ1 was inhibited as a result of inhibition of PI 3-kinase. Crosslinking of FcγRs results in tyrosine phosphorylation of PLC-γ1 that is correlated with IP3 release (Kiener et al., 1993; Shen et al., 1994; Azzoni et al., 1992). However, we found that wortmannin, although inhibiting PLC-γ1 activity, had no effect on PLC-γ1 tyrosine phosphorylation. PI 3-kinase activity was present in PLC-γ1 immunoprecipitates, and this activity was increased upon crosslinking of FcγRIIA. Assay of total PI kinase in p85 immunoprecipitates, however, did not show any change upon FcγRIIA activation. This implies that the pool of PI 3-kinase responsible for PLC-γ1 activation is the rather small fraction of the total PI 3-kinase associated with PLC-γ1. Our data revealed that tyrosine phosphorylation of PLC-γ1 is necessary but not sufficient for its activity.

FcγRIIA doesn't seem to interact with PI 3-kinase directly. Previous results showed that the SH2 domains of p85 regulatory subunit of PI 3 kinase bind to Y-X-X-M motif (Fantl et al., 1992; Kazlauskas et al., 1992), the proline-rich region (residues 84 to 99) of p85 was associated with SH3 domain of activated Lyn and Fyn as a result of ligation of B cell antigen receptor, leading to increased PI 3- kinase activity. CD28, the T cell antigen required for T cell proliferation, bound to PI 3-kinase via its cytoplasmic Y-M-X-M motif (Prasad et al., 1994). In Jurkat T cells,

however, by using synthetic peptide, PI 3-kinase was shown to bind to the first ARAM motif (Y-X-X-L) of the ζ chain, which contains three repeated ARAM motifs (Exley et al., 1994). As crosslinking of GPI-anchored FcγRIIIB in neutrophils also led to activation of PI 3-kinase, it appeared that PI 3-kinase did not bind to activated receptor, rather, it may interact with other signaling molecules such as PLC-γ1 or the putative γ chain.

It is interesting to note that the enzymes participating in lipids metabolism formed a complex (e.g., PLC-γ1, PI kinase, diacylglycerol kinase), and their activities were coordinately and efficient regulated to mediate cell signaling. The roles of PI 3-kinase in GPI-anchored neutrophil FcγRIIIB and diacylglycerol kinase needs further investigation.

Acknowledgments

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Figure 5-1. Inhibition of FcγRIIA-mediated frustrated phagocytosis by 10 nM wortmannin in both P388D$_1$ cells transfected with human FcγRIIA and human neutrophils. Cells were plated on glass coverslips coated with F(ab')$_2$, and activated by adding 1 μg/ml anti-FcγRIIA mAb IV.3 Fab or anti-FcγRIIIB mAb 3G8 Fab at 37^{0}C. **A.** control macrophages; **B.** IV.3 Fab stimulated macrophages; **C.** stimulated macrophages in the presence of 10 nM wortmannin; **D.** control neutrophils; **E.** IV.3 Fab stimulated neutrophils; **F.** 3G8 Fab stimulated neutrophils; **G.** IV.3 Fab stimulated neutrophils in the presence of wortmannin; **H.** 3G8 Fab stimulated neutrophils in the presence of wortmannin.

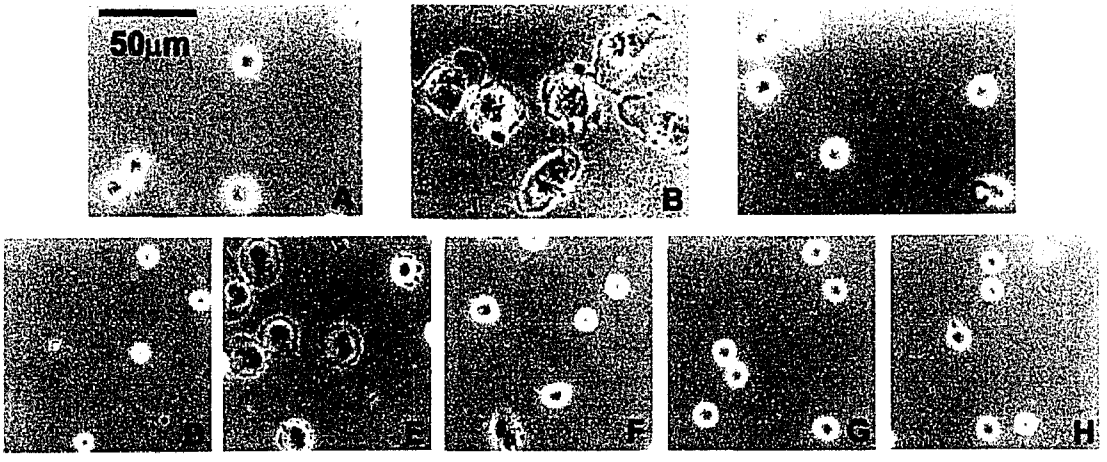


Figure 5-2. Protein tyrosine phosphorylation of PLC-γ1 and p85 regulatory subunit of PI 3-kinase was not altered by wortmannin. Cells were stimulated as detailed in Chapter 2, either in absence of wortmannin, or presence of indicated concentration of wortmannin. Cell detergent lysates were immunoprecipitated with anti-PLC-γ1 IgG and anti-p85 IgG respectively, subjected to SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-phosphotyrosine mAb 4G10.

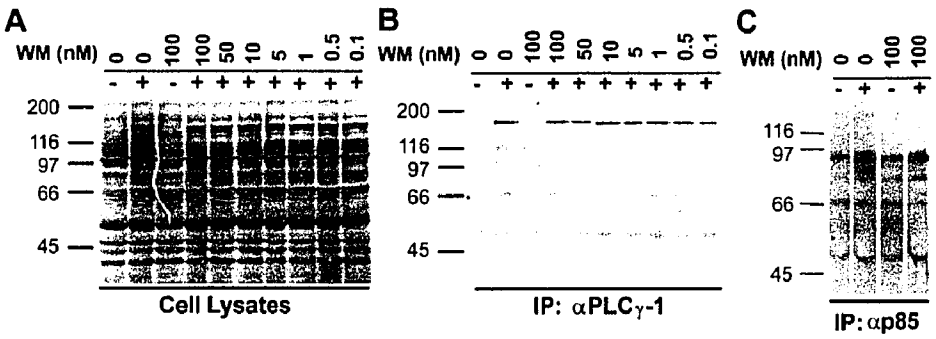


Figure 5-3. Wortmannin inhibited IP3 generation in P388D$_1$ cells transfected with human FcγRIIA. PW16 cells were labeled for two days in medium containing [^{3}H]-myo-inositol. Labeled cells were preincubated either in the presence or in the absence of 10 nM wortmannin for 30 min before stimulation by crosslinking bound IV.3 Fab with goat F(ab')$_2$ anti-mouse IgG. The cells were lysed in 10 mM formic acid, the extracted inositol phosphates were separated by an anion exchange chromatography, and IP3 quantified by a scintillation counter.

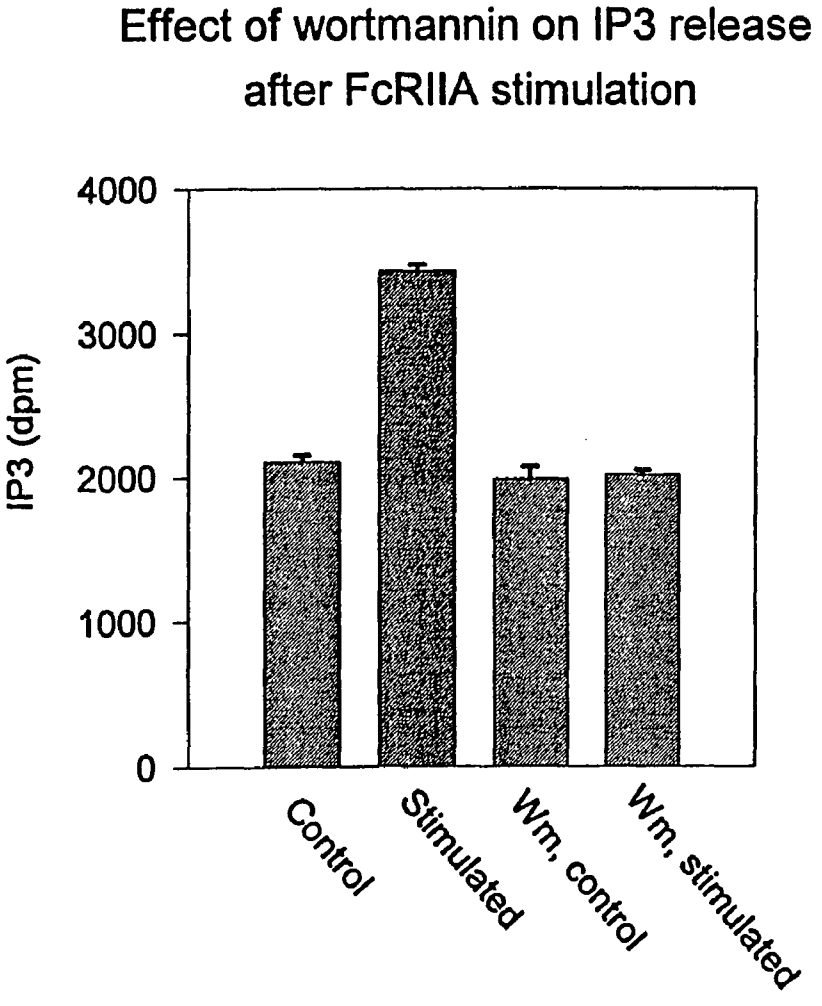
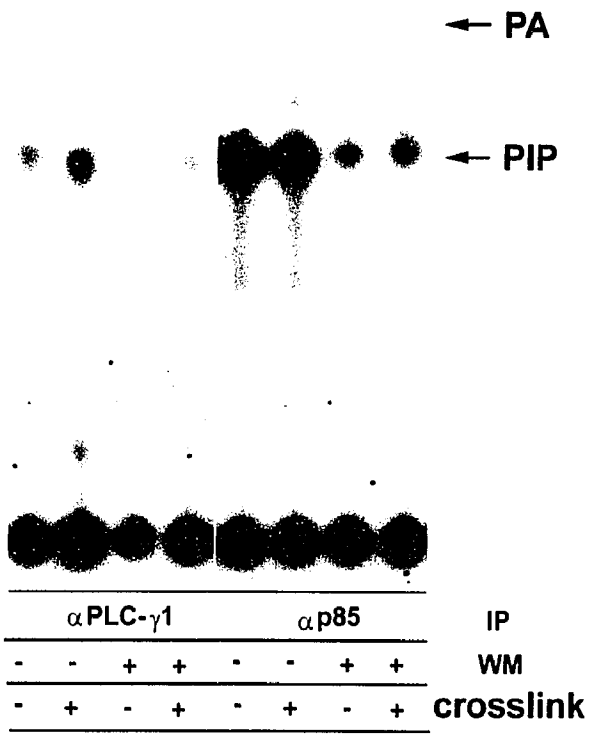


Figure 5-4. PI kinase assay. Macrophages expressing human FcγRIIA were activated as detailed in Chapter 2 with or without wortmannin (10 nM) as indicated. Control (-) and stimulated (+) cells were lysed in NP-40 lysis buffer, and immunoprecipitated with either α-PLC-γ1 IgG (1 μg) or α-p85 antiserum (2 μl). The immune complexes were collected by protein A-agarose beads followed by PI kinase assay as described in Chapter 2. Radioactivity on TLC plates was visualized using a phosphor screen (Molecular Dynamics), and quantified using image quantification software IQ3.3 (Molecular Dynamics).



Chapter 6

General Discussions

Protein tyrosine phosphorylation is an earliest event in FcγRIIA signaling, and Shc, Syk and PLC-γ1 are downstream targets upon activation of FcγRIIA.

Crosslinking of FcγRs results in a number of cellular responses, including activation of protein tyrosine kinase(s), phagocytosis, ans a $[Ca^{2+}]_i$ transient that is required for the oxidative burst (Macintyre et al., 1988). Previous work in our lab demonstrated that the cytoplasmic domain of FcγRIIA was crucial for its signaling, and that endocytosis and $[Ca^{2+}]_i$ transient were triggered by different regions in the cytoplasmic domain (Odin et al., 1991). Crosslinking FcγRIIA in transfected macrophages led to the activation of tyrosine kinase(s), and phosphorylation on tyrosine residues of cellular substrates that were important for FcγRIIA signaling. Identification of these substrates will help to address the mechanisms by which these signaling molecules regulate FcγR-mediated cellular responses. We found that Shc, a SH2 domain containing adaptor protein, Syk, a tyrosine kinase homologous to ZAP-70 found in T cells, and PLC-γ1, one of the phospholipase C isoforms responsible for generating IP3, were heavily phosphorylated on tyrosine residues upon crosslinking of wild type FcγRIIA. However, neither of three proteins were tyrosine phosphorylated upon crosslinking of mutant receptors (Δ264 and Y252F). As shown previously, neither Δ264 and Y252F were able to trigger a $[Ca^{2+}]_i$ transient (Odin et al., 1991)(unpublished results). Our results suggest that tyrosine phosphorylated Shc, Syk, and PLC-γ1 play critical roles in triggering $[Ca^{2+}]_i$ flux, and the tyrosine kinase Syk

phosphorylates Shc and PLC-γ1, since other Src family kinase(s) are activated in Δ264 and Y252F mutants as most of the cellular substrates were still phosphorylated on tyrosine residues. The tyrosine phosphorylated Shc, Syk and PLC-γ1, although important for $[Ca^{2+}]_i$ transient, were not required for endocytosis, since Δ264 mutant had a wild type-phenotype for FcγRIIA-mediated endocytosis (Fig 6-1).

As a model for studying frustrated phagocytosis, FcγRIIA-transfected P388D$_1$ cells were plated onto F(ab')$_2$ goat anti-mouse IgG coated glass plates and then triggering the receptor activation with the addition of mAb IV.3 Fab, a process that mimics *in vivo* phagocytosis. This model provides a tool to maximally activate the FcγRIIA. We demonstrated that the rapid dephosphorylation occurred after crosslinking of FcγRIIA (occur within 5 min) was primarily due to the internalization of the surface receptor., and showed a prolonged kinetics of tyrosine phosphorylation of cellular substrates compared to that cells were activated accompanied by endocytosis. The frustrated phagocytosis model will be potentially very useful for studying protein tyrosine kinase(s) and tyrosine phosphatase(s), since the prolonged kinetics of tyrosine phosphorylation reflect an equilibrium between tyrosine kinase(s) and phosphatase(s) that are maximally stimulated.

Phagocytosis requires protein tyrosine phosphorylation, $[Ca^{2+}]_i$ flux, and the participation of PI 3-kinase

Previous studies in our lab suggested that phagocytosis and endocytosis require different signals (Odin et al., 1991). As discussed above, tyrosine phosphorylation of cellular substrates was necessary for FcγRIIA-mediated phagocytosis, which was consistent with other reports (Greenberg et al., 1993; Greenberg et al., 1994). However, crosslinking the Δ264 mutant FcγRIIA led to tyrosine phosphorylation of a subset of substrates, yet this mutant mediated endocytosis, implicating that all tyrosine kinase(s) were not necessary for endocytosis. Using the model of frustrated phagocytosis, we further demonstrated that phagocytosis and endocytosis required different signals. Protein tyrosine phosphorylation, and $[Ca^{2+}]_i$ transient were absolutely required for phagocytosis, as both tyrosine kinase inhibitors and BAPTA, a intracellular Ca^{2+} chelator, blocked FcγR-mediated phagocytosis. In addition, we found that PI 3-kinase was also critical for phagocytosis. Inhibition of PI 3-kinase by wortmannin resulted in blockade of both FcγRIIA- and FcγRIIIB- mediated frustrated phagocytosis, as well as inhibition of $[Ca^{2+}]_i$ transient stimulated by FcγRIIA crosslinking. The inhibition of $[Ca^{2+}]_i$ transient could result from either the inhibition of PLC-γ1 activity or the desensitization of IP3 receptor due to its serine phosphorylation by PI 3-kinase. To distinguish the two possibilities, we measured the IP3 production and found that the generation of IP3 was inhibited. Since the $[Ca^{2+}]_i$ transient came IP3 that was generated by PLC-γ1 in macrophages, we postulated that PI 3-kinase might associate with PLC-γ1. Indeed, PI 3-kinase activity was found to associate with PLC-γ1, crosslinking of FcγRIIA in transfected macrophages led to

tyrosine phosphorylation of PLC-γ1 and elevated PI 3-kinase activity that could be coprecipitated with PLC-γ1. PI 3-kinase may regulate PLC-γ1 activity through direct serine phosphorylation. Alternatively, PI 3-kinase can regulate PLC-γ1 activity through its lipid products (Fig.6-1).

Although there was PI 3-kinase activity associated with PLC-γ1, it was difficult to detect the subunits of PI 3-kinase in PLC-γ1 immunoprecipitates. The association of PI 3-kinase with PLC-γ1 might occur at low stoichiometry. It will be interesting to isolate the complex between PI 3-kinase and PLC-γ1 by sucrose gradient in order to address this question. It is likely that there is two pools of PI 3-kinase since PI 3-kinase activity was detectable even in cells without any stimulation (Fukui and Hanafusa, 1989). We postulate that the PLC-γ1 associated PI 3-kinase may contain higher specific activity that actually transduces signaling in response to FcγR crosslinking, while the main pool of PI 3-kinase is probably important for maintaining other cell function such as protein sorting (Schu et al., 1993). To address the specific activity of PI 3-kinase, *in vitro* depletion study will performed by doing sequential immunoprecipitation of PLC-γ1 to deplete all PLC-γ1 followed by immunoprecipitation of p85 regulatory subunit of PI 3-kinase, and then assay for PI kinase activity in different immunoprecipitates.

It was reported recently that tyrosine phosphorylated FAK could bind to the SH2 domain of Grb2, linking integrin-mediated signaling to Ras pathway (Schlaepfer, 1994). We found that β-2 integrin was involved in FcγRIIA and FcγRIIIB-mediated

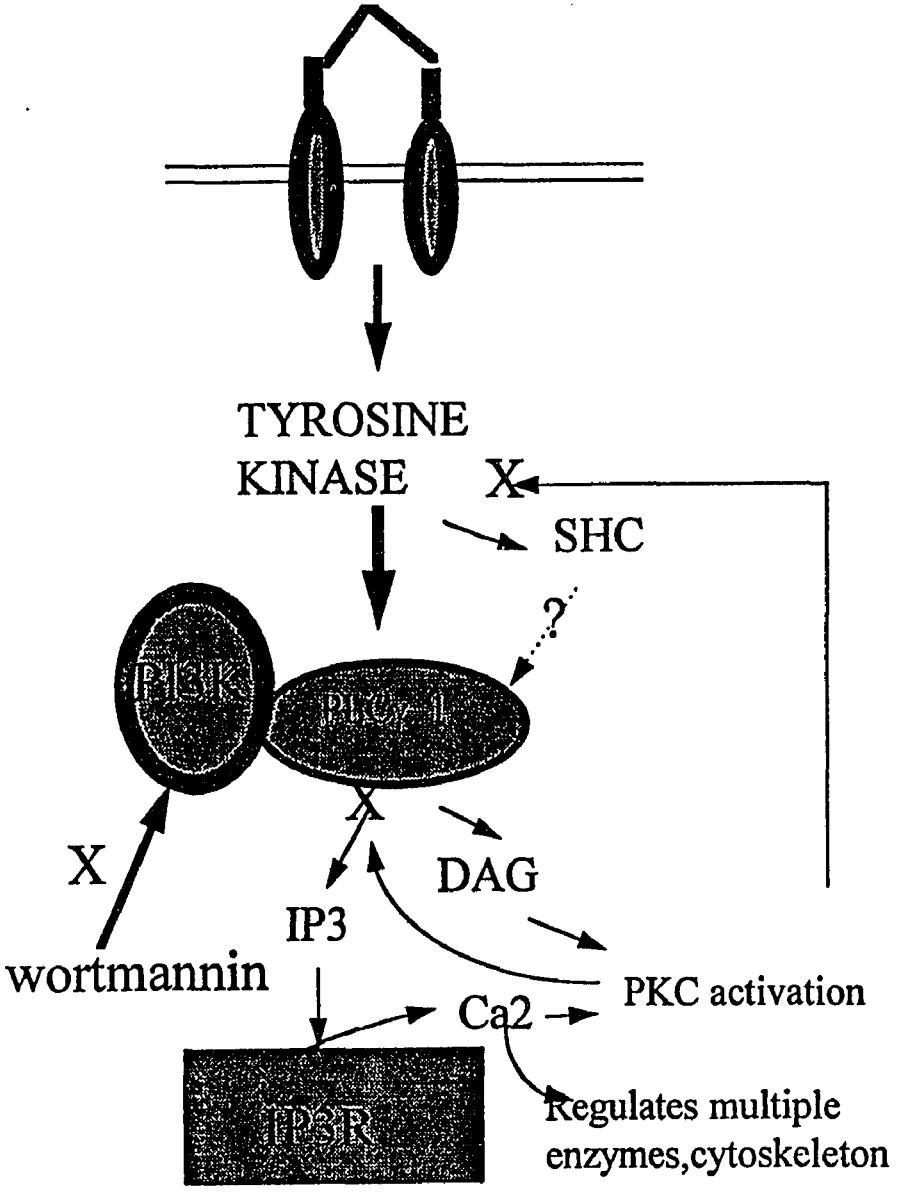
phagocytosis. It is likely that focal adhesion kinase (FAK), whose activity was activated by tyrosine phosphorylation (Guan and Shalloway, 1992), was activated when cells were plated on $F(ab')_2$ goat anti-mouse IgG-coated plates and triggered with Fab of mAb against FcγR leading to massive cell spreading.

Activation of protein kinase C down-regulates FcγRIIA- and FcγRIIIB-mediated signaling

Crosslinking of FcγRIIA in macrophages also resulted in activation of protein kinase C. It was reported that protein kinase C was involved in phagocytosis (Zheleznyak and Brown, 1992) and in focal adhesion formation (Woods and Couchman, 1992). We showed that one of the isoforms, PKC-δ, whose kinase activity correlated with its tyrosine phosphorylation, was phosphorylated on tyrosine residues upon crosslinking of FcγRIIA. However, cells pretreated with phorbol ester (PMA) showed significantly decreased $[Ca^{2+}]_i$ transient after crosslinking of FcγRIIA, suggesting that there was a feedback inhibition mechanism involved in PKC signaling. Crosslinking of FcγRIIA resulted in activation of PLC-γ1 leading to the production of IP3 and DAG, and DAG then activated PKC that may subsequently inhibited the IP3 triggered $[Ca^{2+}]_i$ transient. Thus, activation of PKC desensitized FcγR-mediated signaling. Alternatively, PKC may inhibit $[Ca^{2+}]_i$ transient by phosphorylating the IP3 receptor leading to the desensitization of the IP3 receptor (Ferris et al., 1991). A

number of protein kinase C inhibitors potentiated both Ca^{2+} flux and phagocytosis mediated by FcγRIIA and FcγRIIIB in neutrophils, which was consistent with other report (Svetlov and Nigam, 1993). However, we did not find the same phenomenon in macrophages. It is likely that Fgr , a src-family tyrosine kinase that uniquely expressed in neutrophils but not macrophages (Hamada et al., 1993), may act on PKC to regulate FcγR-mediated phagocytosis, since we found that activation of tyrosine kinases was critical for phagocytosis. Alternatively, different PKC isoforms may be activated in different cells. We showed that PKC-δ was tyrosine phosphorylated and activated in $P388D_1$ cells. However, we were unable to detect tyrosine-phosphorylated PKC-δ in human neutrophils. The different responses of neutrophils and macrophages to calphostin C are probably due to the activation of different PKC isoforms. The role of PKC in FcγR-mediated signal transduction needs further investigation.

Figure 6-1. Model for FcγR-mediated signal transduction.



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