Because hematopoietic cells express multiple Fcγ receptor isoforms, the role of the individual Fcγ receptors in phagocytosis has been difficult to define. Transfection of Fcγ receptors into COS-1 cells, which lack endogeneous Fcγ receptors but have phagocytic potential, has proved valuable for the study of individual Fcγ receptor function. Using this model system, we have established that a single class of human Fcγ receptor mediates phagocytosis in the absence of other Fc receptors and that isoforms from each Fcγ receptor class mediate phagocytosis, although the requirements for phagocytosis differ. In investigating the relationship between structure and function for Fcγ receptor mediated phagocytosis, the importance of the cytoplasmic tyrosines of the receptor or its associated γ chain has been established. For example, two cytoplasmic YXXL sequences, in a configuration similar to the conserved tyrosine-containing motif found in Ig gene family receptors, are important for phagocytosis by the human Fcγ receptor, FcγRIIA. FcγRI and FcγRIIA do not possess cytoplasmic tyrosines but transmit a phagocytic signal through interaction with an associated γ subunit that contains two YXXL sequences in a conserved motif required for phagocytosis. The human FcγRII isoforms FcγRIIB1 and FcγRIIB2 do not induce phagocytosis and have only a single YXXL sequence. Cross-linking the phagocytic Fcγ receptors induces tyrosine phosphorylation of either FcγRIIA or the γ chain, and treatment with tyrosine kinase inhibitors reduces both phagocytosis and phosphorylation of the receptor tyrosine residues. Activation of protein tyrosine kinases follows Fcγ receptor engagement of IgG-coated cells. The data indicate that coexpression of the protein tyrosine kinase Syk, which is associated with the γ chain in monocytes/macrophages, is important for phagocytosis mediated by FcγRI and FcγRIIA. Furthermore, phosphatidylinositol-3 kinase is required for phagocytosis mediated by FcγRIIA as well as for phagocytosis mediated by FcγRI/γ and FcγRIIA/γ.

© 1995 by The American Society of Hematology.

From the Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA.
Submitted February 8, 1995; accepted August 11, 1995.
Address reprint requests to Alan D. Schreiber, MD, The Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.
© 1995 by The American Society of Hematology.
0006-4971/95/8612-0014/$3.00/0

BLOOD
The Journal of
The American Society of Hematology
DECEMBER 15, 1995

REVIEW ARTICLE

The Molecular Dissection of Fcγ Receptor Mediated Phagocytosis

By Zena K. Indik, Jong-Gu Park, Sharon Hunter, and Alan D. Schreiber

ONE OF THE MOST IMPORTANT functions of white blood cells and tissue macrophages is the ingestion or phagocytosis of IgG-coated cells. Receptors for the constant region of IgG, the Fcγ receptors, enable these cells to detect and destroy IgG-coated microorganisms during infection and IgG-coated blood cells in autoimmune disorders.1,2

There are three major classes of Fcγ receptors, designated FcγRI, FcγRII, and FcγRIII. They are encoded by at least 8 genes localized on chromosome 1 at q21-23, and additional isoforms within the three classes are generated through alternative splicing.3,7 The three classes of Fcγ receptors contain highly conserved extracellular Ig domains, but their cytoplasmic regions are distinct from one another, suggesting that they may not all be involved in transmitting a phagocytic signal. Because multiple Fcγ receptor isoforms are expressed in tissue macrophages and other phagocytic cells, it has been difficult to ascertain which Fcγ receptors induce phagocytosis in the absence of other Fcγ receptors and what molecular structures are required.

To study the phagocytic function of these receptors, we sought a model system in which endogeneous Fcγ receptors are not expressed. We found that COS-1 cells, a fibroblast/epithelial-like cell line derived from monkey kidney cells, had sufficient phagocytic machinery to allow phagocytosis when transfected with Fcγ receptor cDNA.8 Analysis of the function of transfected Fcγ receptor cDNAs in COS-1 cells established that a single class of human Fcγ receptor in the absence of other Fc receptors can induce phagocytosis of IgG-sensitized red blood cells (EA; see Fig 1A).5,13 Furthermore, these studies showed that isoforms of all three Fcγ receptor classes are able to transmit a phagocytic signal, although each has particular requirements (see below). In electron micrographs, erythrocytes ingested by Fcγ receptor transfected COS-1 cells appear within well-defined membrane-bound vacuoles, similar to phagocytosis by traditional phagocytes such as macrophages which express endogeneous Fcγ receptors (Fig 1B).8,12,14,15 Degradation of ingested particles is observed within discrete vacuoles and is consistent with the effect of intracellular lysosomal enzymes. The ingestion of EA does not proceed at 0°C and is inhibited by cytochalasin D, which interrupts the assembly of actin filaments essential for phagocytosis.8,10 Sham-transfected cells do not ingest EA, and neither transfected nor untransfected COS-1 cells ingest unsensitized erythrocytes.5,13 That COS-1 cells have the biochemical machinery to support phagocytosis...
Fig 1. Phagocytosis of IgG-sensitized RBCs by Fcy receptor transfected COS-1 cells. (A) Light micrograph of a single COS-1 cell transfected with FcyRIIA and its \( \gamma \) subunit demonstrating the phagocytosis of IgG-sensitized RBCs. (B) Electron micrograph of a COS-1 cell transfected with FcyRIIA is shown. The internalized EA are present within discrete vacuoles. No phagocytosis is observed with sham transfectants or with transfectants incubated with unsensitized RBCs.

Fig 3. Phagocytosis of IgG-sensitized RBCs by CHO cells transfected with FcyRIIA. Transfection was performed by electroporation of FcyRIIA cDNA inserted into the plasmid PRC/CMV. Stable cell lines of FcyRIIA-expressing CHO cells were established by G418 selection.
MOLECULAR DISSECTION OF PHAGOCYTOSIS

Murine γ Chain

FcyRIIA

FcyRIIB WT

B2/YMTL

B2/YMTL/YQNRI

B2/YQNRI

Fig 2. The cytosolic domains of the murine γ chain, FcyRIIA and wild-type and mutant FcyRIIB2 receptors in the region of conserved YXXL sequences. FcyRIIA contains a cytoplasmic region with two YXXL sequences, which is similar to the consensus sequence found in molecules of the Ig gene superfamily implicated in signal transduction. Wild-type human FcyRIIB1 and FcyRIIB2 lack this consensus motif and have only a single cytoplasmic YXXL sequence. YXXL and YXXXI sequences are underlined and in bold face. In the mutant receptor B2/YQNRI, the YXXL sequence is replaced by tyrosine in wild-type FcyRIIB2.

We first observed that an isoform of the low-affinity FcγRII receptor FcyRIIA could mediate a phagocytic signal in COS-1 cell transfectants, whereas the high-affinity Fcy receptor FcyRI could not. FcyRIIA, which requires its γ subunit for receptor expression, required the γ subunit to induce phagocytosis. On the other hand, the FcyRII isoforms, FcyRIIB1 and FcyRIIB2, did not induce phagocytosis. Because these Fcy receptors are characterized by distinctive cytoplasmic domains but similar extracellular regions, their disparities in function further focused our attention on the cytoplasmic domain of the Fcy receptors.

FcγRII

Isoforms of FcγRII arise from the expression of three FcγRII genes, FcyRIIA, FcyRIIB, and FcyRIIC, and alternative splicing of FcγRIIB. Although the extracellular and transmembrane regions of these isoforms are similar or identical, several of these receptors, eg, FcyRIIA, FcyRIIB1, and FcyRIIB2, show variability in the length and structure of their cytoplasmic domains. The cytoplasmic domain of FcγRIIA contains two copies of the conserved tyrosine (Y) containing sequence, YXXL, plus an additional tyrosine residue not in a typical YXXL sequence. Human FcγRIIB1 and FcγRIIB2 contain only a single YXXL sequence in their cytoplasmic domains (Fig 2). Two such YXXL sequences are included in a conserved motif, the ITAM (Ig gene family tyrosine activation motif, DEX, YXXL/IHxYXXL), observed in the cytoplasmic domains of several Ig gene family receptors such as the T-cell and B-cell receptor systems. The sequence of FcγRIIA is similar to this ITAM region.

Human FcγRIIA expressed in transfected COS-1 cells efficiently induces the phagocytosis of EA; however, transfected COS-1 cells, which express a mutant FcγRIIA receptor lacking the cytoplasmic domain, bind EA but do not mediate their phagocytosis. These observations indicated that the cytoplasmic domain of FcγRIIA contains determinants needed for the phagocytosis of IgG-sensitized cells and are consistent with the experiments of Odin et al., which showed that murine macrophage transfectants expressing a truncated FcγRIIA do not phagocytose EA targeted to human FcγRII. Therefore, we hypothesized that the two YXXL sequences in the cytoplasmic domain of FcγRIIA accounted for the ability of FcγRIIA to transmit a phagocytic signal and that the presence of this sequence in the cytoplasmic domain of FcγRIIB1, FcγRIIB2, and FcγRI was responsible for the inability of these receptors to mediate phagocytosis.

Tyrosine phosphorylation of multiple substrates accompanies activation of Fcγ receptors and is required for Fc receptor mediated phagocytosis in mouse macrophages. Cross-linking of FcγRIIA elicited a strong tyrosine phosphorylation response in COS-1 cells transfected with FcγRIIA. A strongly phosphorylated band was observed at 40 Kd in immunoblots derived from antiphosphotyrosine immunoprecipitates of cross-linked FcγRIIA transfectants. This was identified as FcγRIIA in cells that had been surface-labeled with biotin and immunoprecipitated with anti-FcγRII monoclonal antibody (McAb). As in mouse macrophages, inhibitors of tyrosine kinases also decreased phagocytosis in COS-1 cell FcγRIIA transfectants.

COS-1 cells transfected with FcγRIIB1, FcγRIIB2 or, FcγRI also bind large numbers of IgG-sensitized red blood cells (RBCs) externally but, unlike FcγRIIA transfectants, are unable to phagocytose EA. FcγRI has no cytoplasmic tyrosines, but, as noted above, FcγRIIB1 and FcγRIIB2 have a single cytoplasmic YXXL sequence. We examined whether an additional YXXL sequence inserted into FcγRIIB1 and FcγRIIB2 would enable these receptors to transmit a phagocytic signal. Introduction of the membrane proximal YXXL of FcγRIIA, YMTL, into FcγRIIB2 (upstream of the existing YSLL) results in the juxtaposition of two YXXL sequences and the establishment of a motif resembling that found in FcγRIIA (Fig 2). This mutation, B2/YMTL, does not result in full receptor-mediated phagocytic activity as compared with that of FcγRIIA but does allow a low, reproducible level of phagocytosis. A second mutation in FcγRIIB2 that replaces aspartic acid by tyrosine (11 amino acids downstream of the existing YSLL) creates the sequence YXXXI and a motif...
similar to the configuration in murine FcγRIIB2. This mutant, designated B2/YQNRI, also allows some phagocytosis in transfected COS-1 cells. The mutation of aspartic acid to tyrosine to create YXXXI in B2/YMTL results in a configuration containing three tyrosines, similar to the motif in FcγRIIA but with 13 instead of 15 amino acids separating two of the tyrosines (Fig 2). This mutant of FcγRIIB2 (B2/YMTL/YQNRI) mediates more efficient phagocytosis of EA. Thus, insertion of additional YXXL sequences into FcγRIIB2 provides an environment permissive for phagocytosis by FcγRIIB2 and shows that both the number and placement of YXXL sequences in the cytoplasmic domain affect the phagoctytic competence of the FcγRII family of receptors. Furthermore, FcγRIIB2, which does not mediate phagocytosis, is not readily phosphorylated in EA-activated COS-1 cell transfecants, but B2/YMTL/YQNRI transfecants, which support phagocytosis, are phosphorylated on tyrosine after receptor activation. B2/YMTL transfecants that support phagocytosis to a lesser extent are also phosphorylated to a lesser extent.

It is noteworthy that the tissue distribution of the human FcγRII subclasses may also be an indication of their functional differences. FcγRIIB1 and FcγRIIB2, which do not mediate phagocytosis, are expressed in lymphoid cells as well as in other cells of myeloid origin. In contrast, phagocytosis-competent FcγRIIA is expressed in monocytes and neutrophils but is absent from most B-cells or cell lines of B-lymphoid origin not normally associated with Fcγ receptor-mediated phagocytosis.

We next investigated which of the tyrosines in the FcγRIIA cytoplasmic domain are important in phagocytosis and in tyrosine phosphorylation of the receptor itself. Truncation mutations of the cytoplasmic domain that eliminated YXXL sequences inhibited both receptor tyrosine phosphorylation and receptor-induced phagocytosis. Replacement of the first cytoplasmic tyrosine, Y1 (which is not within a typical YXXL motif), by phenylalanine (Y1F) did not reduce phosphorylation, whereas substitution of the second or third cytoplasmic tyrosine, Y2 or Y3 (both within YXXL sequences; see Fig 2), with phenylalanine substantially inhibited but did not eliminate phagocytosis. Replacement by phenylalanine of any two tyrosines, including combinations of Y1F, resulted in an essentially complete loss of phagocytic activity. These data suggest that Y2 and Y3, which are within YXXL sequences, and/or the structure of these domains are particularly important for the phagocytic activity of FcγRIIA. Although the role of the first cytoplasmic tyrosine of FcγRIIA (Y1), which is not within a typical YXXL sequence, is uncertain, it also appears to contribute to phagocytic function and may play a "backup" role in the absence of Y2 or Y3.

Tyrosine phosphorylation of FcγRIIA was different for each Y to F replacement mutant, and there was not a simple relationship between phagocytosis and phosphorylation of the FcγRIIA cytoplasmic tyrosines. All single tyrosine mutants showed reduced induction of tyrosine phosphorylation; however, the most severe reduction in tyrosine phosphorylation was observed for Y3F and for mutants in which 2 of the 3 tyrosines were substituted by phenylalanine. Thus, the most downstream tyrosine (Y3) appears to be particularly important, because its removal by truncation or its replacement with phenylalanine inhibits tyrosine phosphorylation and phagocytosis in parallel.

Residues other than the cytoplasmic tyrosines also appear to play a role in these receptor functions. Deletion of the threonine and leucine residues within the conserved YXXL sequences or alterations in the 12 residue proline-containing sequence between the two YXXL motifs also reduced phagoctytic activity and tyrosine phosphorylation of the receptor. Thus, the specific structure of the FcγRIIA cytoplasmic domain accounts for its ability to stimulate phagocytosis in the absence of other receptor subunits.

In further studies, we have shown that FcγRIIA is phosphorylated in vitro by the Src-family tyrosine kinase (SRTK) Src, suggesting that in some hematopoietic cells Src may be involved in phosphorylation of FcγRIIA after cross-linking of the receptor. To determine whether Src is required for FcγRIIA mediated phagocytosis and for phosphorylation of FcγRIIA, we introduced FcγRIIA into a mouse embryonic fibroblast cell line that lacks Src kinase activity. Although both Src-negative cells and wild-type mouse fibroblast Src-positive cells phagocytosed EA, the Src-negative cells were less efficient in mediating phagocytic function. Cross-linking of FcγRIIA with EA resulted in tyrosine phosphorylation of the 40-kD band in both cell lines, suggesting that a tyrosine kinase other than Src also is able to phosphorylate FcγRIIA in vivo.

We have observed that other epithelial-derived cell lines also have the potential for phagocytic function. FcγRIIA transfecants of Chinese hamster ovary (CHO) cells express high levels of the receptor and are able to phagocytose large numbers of EA. In these cells, as with FcγRIIA-transfected COS-1 cells, ingestion of EA is sensitive to incubation with cytochalasin D and does not occur at 4°C.

These studies suggested that other nonphagocytic cells (eg, nonphagocytic cells of hematopoietic lineage) might assume phagocytic properties after transfection of an appropriate Fcγ receptor. Expression of FcγRIIA in T-cells also conferred the ability to mediate IgG-stimulated phagocytosis, and cross-linking FcγRIIA with anti-FcγRII MoAb in these cells induced tyrosine phosphorylation of multiple proteins including FcγRIIA itself. Thus, when transfected into T-cells, FcγRIIA can interact with the T-cell signaling machinery to establish phagocytic function.

Recent studies of the role of the cytoplasmic domain in human and murine Fcγ receptor mediated endocytosis of small molecular weight immune complexes have shown that human FcγRIIA and human and murine FcγRIIB2 transfecants are able to mediate immune-complex endocytosis and that this function requires only short stretches of the receptor cytoplasmic domain. Although phagocytosis and endocytosis are related processes, it appears that fundamental differences exist in the requirements for internalization by these routes. For example, receptor-mediated endocytosis involves localization of immune complexes to clathrin-coated pits, whereas internalization by phagocytosis is dependent on intact actin microfilaments. Therefore, there are cytosolic sequences responsible for phagocytosis which are probably distinct from those required for endocytosis.
Molecular Dissection of Phagocytosis

**FcyRII**

The class III Fcγ receptor FcyRIIIA is expressed as a multichain complex consisting of a single α chain containing IgG-binding domains and a disulfide-linked homodimer or heterodimer consisting of γ and ζ subunits. Association of FcyRIIIA α with the subunits γ and/or ζ occurs through interactions between their transmembrane regions. FcyRIIIA/α is found in macrophages, and both homodimeric and heterodimeric forms of FcyRIIIA occur in natural killer cells that express both the γ and ζ subunits.

In transfected COS-1 cells, FcyRIIIA mediates a phagocytic signal in the absence of any other Fcγ receptor but requires coexpression of the γ subunit for both the surface expression of FcyRIIIA and for transduction of a phagocytic signal. Truncation of the cytoplasmic domain of the γ subunit eliminates phagocytic function, and replacement of the murine γ chain cytoplasmic tyrosine residues with phenylalanine, singly or in pairs, shows that both murine γ chain tyrosine residues are essential for phagocytosis by FcyRIIIA.

Phagocytosis mediated through the γ chain was similarly abolished in mast cells stably transfected with an FcyRIIIA/γ chain chimera in which one or both tyrosine residues of the murine γ chain cytoplasmic domain were mutated.

The ζ chain of the T-cell receptor contains sequences homologous to the γ chain, including the conserved YXXL sequences, in its cytoplasmic region. FcyRIIIA also mediates a phagocytic signal through the ζ chain in FcyRIIIA/ζ chain cotransfectants. However, the ζ chain is less than sixfold as efficient in mediating phagocytosis by FcyRIIIA than is the γ chain. This is of interest because phagocytic monocytes/macrophages express FcyRIIIA in association with the γ chain but not in association with the ζ chain. Exchange mutants suggest that functional differences between the two related subunits γ and ζ are mainly accounted for by the internal amino acids of the YXXL sequence and their paired presence in the ITAM.

In vitro kinase assays using lysates of cultured monocyes indicate that the FcyRIIIA γ chain is phosphorylated on tyrosine residues after FcyRIIIA cross-linking. Furthermore, treatment with the tyrosine kinase inhibitor tyrphostin 23 severely reduces phagocytosis, and phosphorylation of the tyrosine residues of the γ chain appears to correlate with phagocytic capacity in both immunoblotting and in vitro kinase assays. In contrast to the situation with FcyRIIA, in which replacement of a single tyrosine does not completely abolish phagocytic function, replacement of either cytoplasmic tyrosine of the murine γ chain with phenylalanine eliminates both phagocytosis and tyrosine phosphorylation.

Human neutrophils express the FcyRIII receptor, FcyRIIB. FcyRIIB is a phosphatidylinositol glycan-linked Fcγ receptor and lacks transmembrane and cytoplasmic domains. However, it is capable of participating in transmembrane signaling events such as calcium release, neutrophil degranulation and actin polymerization. The mechanism(s) by which FcyRIIB transmits signals is currently being examined. Recent studies have shown that transfected fibroblasts expressing both FcyRIIB and the type-3 complement receptor (CR3) are able to bind and phagocytose IgG-coated RBCs, whereas cells expressing either CR3 or FcyRIIB alone are unable to trigger phagocytosis of E.A. Recent studies have also shown a close physical relationship between CR3 and FcyRIIB in neutrophils and transfected fibroblasts, and it has been suggested that the lectin-like interactions between CR3 and FcyRIIB activate a pathway to generate phagocytosis in these cells. The mechanism(s) by which such lectin-like interactions stimulate phagocytic signaling by the phosphatidylinositol glycan-linked Fcy receptor is not yet understood. Other studies suggest that neutrophil FcyRIIB may stimulate phagocytosis by interacting with FcyRIIB. Such interactions of FcyRIIB with neutrophil CR3 and/or FcyRIIA may provide additional mechanism(s) through which Fcyγ receptors mediate a phagocytic signal.

**FcyRI**

FcyRI is unique among the Fcγ receptors in being confined to resting cells of a single lineage, the phagocytic monocyte/macrophage. COS-1 cell transfectants of FcyRI do not mediate phagocytosis despite avid binding of IgG-coated RBCs. However, phagocytosis mediated through FcyRI was observed in monocytes/macrophages that express multiple Fcγ receptors. Phosphorylation of multiple substrates occurs after cross-linking of FcyRI on monocytes and hematopoietic cell lines, and the tyrosine kinase inhibitors tyrphostin 23 and genistein inhibit FcyRI mediated phagocytosis. Thus, despite the absence of tyrosines in the cytoplasmic domain of FcyRI, protein tyrosine phosphorylation is important for FcyRI mediated phagocytosis. These observations further suggested that a cell-specific gene product present in hematopoietic cells such as macrophages but absent in COS-1 cells was required for FcyRI mediated phagocytosis. To investigate this issue, we prepared stable transfectants of human FcyRI in the murine macrophage cell line P388D1. These human FcyRI transfectants were able to phagocytose RBCs specifically targeted to human FcyRI (E-MoAb). Stable transfectants of a mutant FcyRI lacking the cytoplasmic domain also supported the phagocytosis of E-MoAb in murine macrophages, indicating that the cytoplasmic domain of FcyRI was not required for this process.

The requirement for the γ subunit in FcyRIIIA mediated phagocytosis suggested that the γ chain might also be an accessory molecule, present in macrophages but absent in COS-1 cells, that is required for FcyRI mediated phagocytic signaling. Coexpression of the γ subunit with either wild-type FcyRI or an FcyRI mutant lacking the cytoplasmic domain permitted FcyRI mediated phagocytosis in COS-1 cells. These results are consistent with observations of FcyRI phagocytic function in murine macrophages and with the finding that FcyRI and the γ chain are associated in monocytes and macrophage-like cell lines.

FcyRI has a substantial cytoplasmic domain of 61 amino acids, and, despite the absence of a tyrosine-containing consensus motif in the cytoplasmic domain, FcγRI mediates a Ca^2+ signal in COS-1 cell transfectants in the absence of...
the γ chain.\textsuperscript{64,65} However, in the absence of its cytoplasmic domain, FcγRI does not transmit a Ca\textsuperscript{2+} signal in COS-1 cell transfectants.\textsuperscript{64,65} Thus, it appears that the FcγRI cytoplasmic domain is required for some cellular responses but not for others.

### PROTEIN TYROSINE KINASES

Although isoforms of each class of Fcγ receptor are able to induce the phagocytosis of IgG-coated cells,\textsuperscript{6,14,22,71} the data indicate that their mechanisms for phagocytosis differ. For example, FcγRIIA requires tyrosines within the conserved motif of its own cytoplasmic domain,\textsuperscript{11} whereas both FcγRI and FcγRIIIA require the tyrosines within the conserved cytoplasmic region of the γ subunit for phagocytosis.\textsuperscript{10,13,68} Furthermore, in contrast to the γ chain mediated phagocytosis, some phagocytosis by FcγRIIA is retained in the presence of a single intact YXXL sequence.\textsuperscript{11} In contrast to FcγRI and FcγRIIIA, phagocytosis induced by FcγRIIA occurs efficiently in COS-1 transfectants in the absence of the cotransfection of the γ chain (or Syk kinase, see below).\textsuperscript{8,11} Agents capable of activating protein kinase C such as phorbol esters (e.g., phorbol myristate acetate) have been observed to amplify phagocytosis. Phorbol myristate acetate decreases phagocytosis in transfectants expressing FcγRIIA and the γ chain but modestly increases FcγRII mediated phagocytosis.\textsuperscript{11,17,65} The internal amino acids of the YXXL sequences differ in FcγRIIA and the γ chain, and FcγRIIA has a unique ITAM that contains 12 amino acids, comparable receptor cell surface expression, FcγRIIa concomitant with FcγRII, and FcγRIIα has a unique ITAM that contains 12 amino acids, comparable with the FcγRIIIA and FcγRIIIα.

In the absence of the γ chain, Syk does not induce phagocytosis by either FcγRII or FcγRII. Instead, Syk dramatically enhances phagocytosis mediated by FcγRII and FcγRII. In the absence of the γ chain, Syk dramatically enhances phagocytosis mediated by FcγRII and FcγRII. However, in the absence of its cytoplasmic domain, FcγRII does not transmit a Ca\textsuperscript{2+} signal in COS-1 cell transfectants.\textsuperscript{64,65} Thus, it appears that the FcγRI cytoplasmic domain is required for some cellular responses but not for others.

### PROTEIN TYROSINE KINASES

Although isoforms of each class of Fcγ receptor are able to induce the phagocytosis of IgG-coated cells,\textsuperscript{6,14,22,71} the data indicate that their mechanisms for phagocytosis differ. For example, FcγRIIA requires tyrosines within the conserved motif of its own cytoplasmic domain,\textsuperscript{11} whereas both FcγRI and FcγRIIIA require the tyrosines within the conserved cytoplasmic region of the γ subunit for phagocytosis.\textsuperscript{10,13,68} Furthermore, in contrast to the γ chain mediated phagocytosis, some phagocytosis by FcγRIIA is retained in the presence of a single intact YXXL sequence.\textsuperscript{11} In contrast to FcγRI and FcγRIIIA, phagocytosis induced by FcγRIIA occurs efficiently in COS-1 transfectants in the absence of the cotransfection of the γ chain (or Syk kinase, see below).\textsuperscript{8,11} Agents capable of activating protein kinase C such as phorbol esters (e.g., phorbol myristate acetate) have been observed to amplify phagocytosis. Phorbol myristate acetate decreases phagocytosis in transfectants expressing FcγRIIA and the γ chain but modestly increases FcγRII mediated phagocytosis.\textsuperscript{11,17,65} The internal amino acids of the YXXL sequences differ in FcγRIIA and the γ chain, and FcγRIIA has a unique ITAM that contains 12 amino acids, rather than the prototypic 7 amino acids, separating the two YXXL sequences of the γ chain. These distinct sequences may account for the differences in the signaling pathway(s) used for phagocytosis by FcγRIIA and by the other phagocytic receptors FcγRII/γ and FcγRIIIA/γ.

Although FcγRI, FcγRIIA, and FcγRIIIA clearly mediate phagocytosis in human cultured monocytes and macrophages with similar efficiencies, in COS-1 cells, even with comparable receptor cell surface expression, FcγRIIA consistently mediates higher levels of phagocytosis than FcγRIIIA/γ and FcγRII/γ.\textsuperscript{6,14,22,71,65} The key observation was that COS-1 cells lack another element(s) present in cells of monocyte/macrophage lineage that optimizes γ chain mediated phagocytosis but is not necessary for efficient FcγRIIIA mediated phagocytosis.

One possible candidate for this monocyte/macrophage factor was the protein tyrosine kinase Syk.\textsuperscript{68} Syk is present in hematopoietic cells\textsuperscript{69,70} and communoprecipitates with the γ chain associated with FcγRIIIA in macrophages and with FcγRI in mast cells.\textsuperscript{71,72} Syk is also phosphorylated on tyrosine after cross-linking of FcγRI or FcγRIIIA on cells of the monocyte/macrophage lineage.\textsuperscript{28,30,71} Syk dramatically enhanced the phagocytosis of EA by both FcγRIIIA/γ and FcγRII/γ in transfected COS-1 cells (Fig 4).\textsuperscript{68} For example, for FcγRIIIA/γ the phagocytic index (PI: the number of ingested RBCs per 100 Fcγ receptor expressing cells) was increased from 221 ± 40 to 952 ± 142. Syk also increased the percentage of cells able to phagocytose EA (from 7.7% ± 1% to 22.5% ± 2% of transfectants for FcγRIIIA/γ). Because 25% to 35% of transfectants expressed Fcγ receptors, this indicates that the presence of Syk enables the majority of transfectants to phagocytose EA. Thus the protein tyrosine kinase Syk dramatically enhances phagocytosis mediated by FcγRIIIA and FcγRI and also allows some previously nonphagocytic FcγRI or FcγRIIIA receptor expressing cells to acquire phagocytic capability.

In the absence of the γ chain, Syk does not induce phagocytosis by either FcγRIIIA or FcγRI, and, consistent with the concept that the effect of Syk requires sequences in the γ chain, the cytoplasmic domain of neither FcγRIIIA nor FcγRI is required for the stimulation of phagocytosis by Syk (Fig 4).\textsuperscript{68} These data indicate that Syk markedly enhances the phagocytic signal in two Fcγ receptors associated with the γ chain and shows that introduction of a specific tyrosine kinase can induce a physiologically important cellular function.

Cross-linking of FcγRI in COS-1 transfectants expressing FcγRI, the γ chain, and Syk increases tyrosine phosphorylation.
MOLECULAR DISSECTION OF PHAGOCYTOSIS

Coexpression of Syk with the  chain and either FcyRI or FcyRIIIA also enhances  chain mediated phagocytosis but does not increase the level of phagocytosis to that of the  chain (Fig 4). This result is consistent with previous observations that indicate that the  chain is less efficient than the  chain in inducing phagocytosis in transfected COS-1 cells.15

There is increasing evidence that Syk engages at least two functional SH2-binding domains in interactions with the  chain and that both SH2 regions of Syk are important for the binding of Syk to the  subunit in vitro.74 These data are consistent with our observations in mapping which Fcy receptor sequences are necessary for phagocytosis induced by Syk kinase.66 Syk is unable to induce either FcyRI or FcyRIIIA mediated phagocytosis by  chain mutants in which one  chain YXXL tyrosine, Y1 or Y2, is replaced by phenylalanine (FcyRII/γY1F, FcyRII/γY2F, FcyRIIIA/γY1F, and FcyRIIIA/γY2F; see Fig 4).66 In addition, there is no induction of phagocytosis by Syk in a  chain truncation mutant (γΔ65) that removes the carboxy terminal YXXL-containing region, whereas enhancement of phagocytosis by Syk is observed with a  chain mutant (γΔ80) that only lacks residues downstream of the YXXL sequences.

In an additional  chain mutant (γ-FcyRIIIA) the sequence separating the two cytoplasmic YXXLs of the  chain was replaced by the 12 amino acid-intervening sequence from FcyRIIIA. This lengthened the sequence between the two  chain YXXLs from 7 to 12 amino acids, thus creating a  chain mutant in which the number of amino acids separating the two YXXLs was increased by 5 amino acids. Syk stimulates the phagocytic efficiency of this mutant as well as that of a  chain mutant in which 4 amino acids are deleted from the sequence separating the two YXXLs (γΔ71-73).66 Thus, a sequence of between 3 and 12 amino acids between the two conserved  chain YXXL sequences allows Syk to function in Fcy receptor/chain mediated phagocytosis.

The induction of Syk phosphorylation after cross-linking of FcyRII in monocytes/macrophages32,67 suggested that Syk may also play a role in phagocytosis by FcyRIIA. Syk modestly increased the efficiency of phagocytosis by FcyRIIA and, similarly, modestly increased phagocytosis by the chimeric receptor I-IIA-IIA (EC-TM-CYT), which contains the cytoplasmic domain (CYT) and transmembrane domain (TM) of FcyRIIA and the extracellular domain (EC) of FcyRI (Fig 4).

The use of chimeric receptors also showed the different effects of Syk on FcyRIIIA and  chain induced phagocytosis. Association of the  chain with FcyRIIIA occurs through the TM of FcyRIIIA,66,67 Greater enhancement of phagocytosis by Syk in the presence of the  chain was observed for the chimera I-I-IIA than for either FcyRIIIA or the chimeric receptor I-I-IIA-IIA (Fig 4),66 which is consistent with the thesis that association of FcyRI with the  chain also occurs through its TM.15-17 It is likely that association with the  chain occurs through the FcyRI derived TM of I-I-IIA and that recruitment of the  chain allows a larger Syk phagocytic response by this chimera.

Similar to Syk, the protein kinases of the Src family (SRTKs) Lyn, Fyn, Fgr, Lck, and Src are expressed in phago-

Fig 5. Antiphosphotyrosine immunoblots of stable Syk kinase COS-1 cell transfectants. COS-1/Syk stable transfectants were transfected with FcyRI alone (lane 2), with FcyRI and the  chain (lane 3), or with the  chain alone (lane 4). Lane 1 presents the profile of sham transfectants. After incubation with EA, cells were lysed and immunoprecipitated with anti-Syk antibody. In the presence of the receptor cross-linking enhances tyrosine phosphorylation of Syk, suggesting that Syk is activated under these conditions.66 This result is consistent with the observations in monocytes and macrophages which showed that Fcy-receptor cross-linking enhances tyrosine phosphorylation of Syk kinase.32,33,40,70,71 We have prepared COS-1 cells stably transfected with Syk kinase. In these COS-1 cells (COS-1/Syk), little phosphorylation of Syk occurs in the absence of the  chain, with or without expression of FcyRI (Fig 5). When COS-1/Syk cells were transfected with  chain alone or with FcyRI and the  chain, phosphorylation of Syk was intense, whereas, in COS-1/Syk cells transfected with FcyRI alone, Syk phosphorylation was not observed. This effect occurred in the absence of receptor cross-linking (Fig 5). Thus,  chain expression alone appears sufficient to induce phosphorylation of Syk in these COS-1 cell transfectants. These data suggest that Syk and the  chain are associated endogenously, leading to Syk phosphorylation. Furthermore, taken together,66,71 our data suggest that receptor cross-linking leads to an alteration in the  chain/Syk complex that induces Syk kinase activation.
cytic cells such as monocytes/macrophages. In contrast to the effect of Syk, cotransfection of the Src family tyrosine kinases (SRTKs) Fgr, Fyn, Lyn, Lck, or Src did not increase FcyRIIIA/γ or FcyRI/γ phagocytosis. Nor did these SRTKs further increase the level of phagocytosis induced by Syk. The observation that of these protein kinases, only Syk kinase enhanced phagocytosis by FcyRI or by FcyRIIA in the presence of the γ chain suggests a specificity of Syk for γ-chain sequences. The low levels of FcyRI/γ and FcyRIIIA/γ mediated phagocytosis in COS-1 cells in the absence of transfected Syk may be due to endogenous tyrosine kinases, which are less efficient than Syk in interactions with γ chain sequences, or to low levels of Syk that may be present in some COS-1 cells. It is not yet known whether endogenous COS-1 cell Src family kinases (SRTKs) are necessary for Syk’s effect or whether Syk-induced phagocytosis in the presence of the γ chain is independent of SRTKs.

The protein kinase ZAP-70 is homologous to Syk kinase and has been shown to associate with the ζ chain in the T-cell–antigen complex. In contrast to Syk, the related kinase ZAP-70 did not stimulate FcyRIIIA mediated phagocytosis in transfected COS-1 cells. However, ZAP-70 increased phagocytosis when coexpressed with the Src kinase Fyn. Thus, although related structurally, ZAP-70 and Syk kinases differ in their requirements for an Src-related kinase in phagocytic signaling.

Phosphatidylinositol-3 (PI-3) kinase has been implicated in the signaling pathways of a number of receptor systems. For example, cross-linking of natural killer cell FcyRIII or the T-cell receptor initiates activation of protein tyrosine kinases and induction of a PI-3 kinase-dependent pathway. We studied the effect of wortmannin, a specific inhibitor of PI-3 kinase, on Fcy receptor mediated phagocytosis. In COS-1 cell transfectants, wortmannin inhibited Fcy receptor mediated phagocytosis in a dose-dependent fashion, with virtually complete inhibition of phagocytosis occurring at 25 nmol/L (Figs 6A and B), a concentration of wortmannin that specifically inhibits PI-3 kinase and not PI-4 kinase.

This effect was observed for FcyRIIIA mediated phagocytosis as well as for γ chain dependent phagocytosis mediated through FcyRI/γ and FcyRIIIA/γ and was comparable for each Fcy receptor (Fig 6B). The IC₅₀ for inhibition of Fcy receptor mediated phagocytosis by wortmannin was ~8 nmol/L, a value consistent with the PI-3 kinase inhibitor concentrations reported to inhibit other Fc receptor cellular responses. In contrast, concentrations of bisindolyl-maleimide, which completely inhibit the activity of protein kinase C, did not inhibit Fcy receptor mediated phagocytosis (data not shown). These observations indicate that PI-3 kinase is essential for phagocytosis mediated by each of the phagocytic Fcy receptors.

We have also examined the relationship of PI-3 kinase and Syk in Fcy receptor transfectants. A phosphorylated band of ~72 kD that comigrates with Syk is immunoprecipitated by anti–PI-3 kinase from lysates of cross-linked FcyRI transfectants coexpressing FcyRI, the γ chain, and Syk (Fig 7). In addition, after Fcy receptor cross-linking, anti-Syk coprecipitates a band that migrates at a position comparable with that of PI-3 kinase. These observations suggest that PI-3 kinase and Syk are associated in vivo. Because it has been observed that PI-3 kinase binds to the SH3 domains of Src family-related tyrosine kinases, the association of PI-3 kinase with Syk may occur through a complex of Syk and a Src-related tyrosine kinase(s). However, no evidence of a Src-related tyrosine kinase has thus far been observed in our immunoprecipitates, suggesting that Syk directly associates with PI-3 kinase.
MOLECULAR DISSECTION OF PHAGOCYTOSIS

In summary, isoforms of each class of Fcγ receptor are able to induce the phagocytosis of IgG-coated cells.\cite{8,13,27,30,31} Furthermore, phagocytic function may be induced in certain nonphagocytic cells by the addition of appropriate Fcγ receptors.\cite{8,13,27,30,31} Although phagocytosis mediated through FcγRIIA as well as through FcγRI/γ and FcγRIIIA/γ requires PI-3 kinase activity, in other respects, the mechanisms for Fcγ receptor mediated phagocytosis differ. FcγRIIA induces phagocytosis associated with the phosphorylation of tyrosines within the conserved motif of its own cytoplasmic domain,\cite{8,13,27,30,31} whereas both FcγRI and FcγRIIIA require the tyrosines within the conserved cytoplasmic region of their γ subunit for phagocytosis.\cite{10,13,27,30,31} The protein tyrosine kinase Syk markedly enhances the phagocytic signal in the two Fcγ receptors associated with the γ chain.\cite{8,13,27,30,31} Enhancement of FcγRIIA mediated phagocytosis by Syk kinase is modest compared with that of FcγRI/γ mediated or FcγRIIIA/γ mediated phagocytosis. Efficient phagocytosis by transfected FcγRIIA is mediated by the endogeneous kinases of COS-1 cells (presumably Src family kinases) in the absence of cotransfected Syk kinase.\cite{8,13,27,30,31} The data suggest that the unique ITAM in FcγRIIA may account for the differences in signal transduction between FcγRIIA and the γ associated phagocytic receptors FcγRI and FcγRIIIA. The data further suggest that Syk kinase and the γ subunit of FcγRI and FcγRIIIA are associated endogenously, leading to initial Syk tyrosine phosphorylation in the absence of Fcγ receptor cross-linking.

ACKNOWLEDGMENT

We acknowledge the contribution of Xiao Qing Pan to these experiments.

REFERENCES

4. McKenzie SE, Schreiber AD: Biological advances and clinical applications of Fc receptors for IgG. Curr Opin Hematol 1:45, 1994
15. Kruskal BA, Sastry K, Warner AB, Mathieu CE, Ezekowitz RAB: Phagocytic chimeric receptors require both transmembrane

![Fig 7. Antiphosphotyrosine immunoblots of FcγRI/γ/Syk COS-1 cell transfectants. Lanes 1 and 3 are sham transfectants, and lanes 2 and 4 are COS-1 cells transfected with FcγRI, the γ chain subunit, and Syk. Lanes 1 and 2, anti-Syk immunoprecipitates; lanes 3 and 4, anti–PI-3 kinase immunoprecipitates. Molecular weight markers are indicated on the left. The arrows indicate the position of Syk, and the asterisks indicate the position of PI-3 kinase. PI-3 kinase was identified by immunoblot in a parallel experiment.](image-url)


32. Kiener PA, Rankin BM, Burkhardt AL, Schieven GL, Gilliland LK, Rowley RB, Bolen JB, Ledbetter JA: Crosslinking of Fcγ receptor I (FcγRI) and receptor II (FcγRII) on monocytes activates a signal transduction pathway common to both Fc receptors that involves the stimulation of p72 Syk protein tyrosine kinase. J Biol Chem 268:24442, 1993.


61. Ernst LK, Duchemin A-M, Anderson CL: Association of the high affinity receptor of IgG (FcγRI) with the γ subunit of the IgE receptor. Proc Natl Acad Sci USA 90:6023, 1993
63. Scholl PR, Geha RS: Physical association between the high affinity receptor for IgG (FcγRI) and the γ subunit of the high affinity IgE receptor (FcεRI). Proc Natl Acad Sci USA 90:8847, 1993
65. Indik Z, Chien P, Levinson AI, Schreiber AD: Calcium signaling by the high affinity macrophage Fcγ receptor requires the cytosolic domain. Immunobiology 185:183, 1992
67. Kelly CJ, Indik ZK, Isaacs R, Chien P, Daly JM, Schreiber AD: The human platelet/megakaryocyte Fcγ receptor (FcγRI) mediates a phagocytic signal which is enhanced by phorbol ester activation. Blood 78:393a, 1991
72. Hutchcroft JF, Geahlen RL, Deanin GG, Oliver JM: Fc epsilon RI-mediated tyrosine phosphorylation and activation of the 72 kDa protein kinase, PTK72, in RBL-2H3 rat tumor mast cells. Proc Natl Acad Sci USA 89:9107, 1992
The molecular dissection of Fc gamma receptor mediated phagocytosis

ZK Indik, JG Park, S Hunter and AD Schreiber