BLOOD

The Journal of
The American Society of Hematology

VOL 86, NO 12 DECEMBER 15, 1995

REVIEW ARTICLE

The Molecular Dissection of Fcγ Receptor Mediated Phagocytosis

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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 FcγRIIIA 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 FcγRIIIA. Furthermore, phosphatidylinositol-3 kinase is required for phagocytosis mediated by FcγRIIA as well as for phagocytosis mediated by FcγRI/γ and FcγRIIIA/γ.

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).[8-13] 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 endogenous Fcγ receptors (Fig 1B).[9,12,14,15] 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.[8,16] Sham-transfected cells do not ingest EA, and neither transfected nor untransfected COS-1 cells ingest unsensitized erythrocytes.[8-13] That COS-1 cells have the biochemical machinery to support phagocy-

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Submitted February 8, 1995; accepted August 11, 1995.

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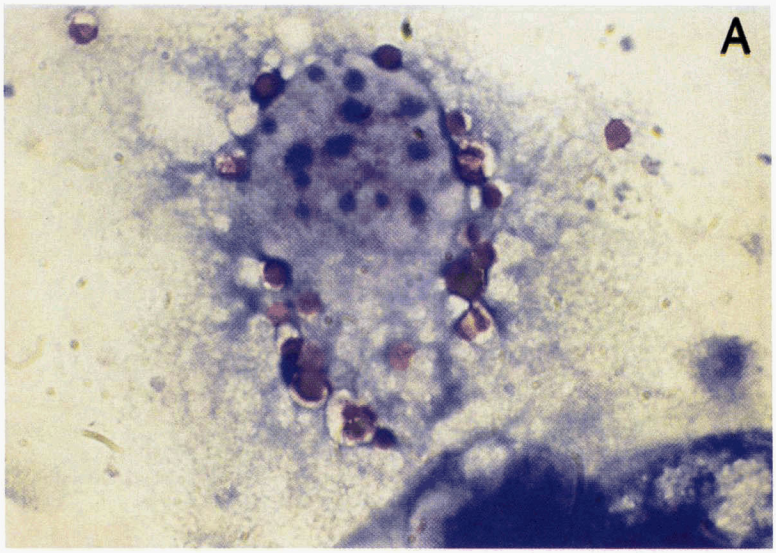

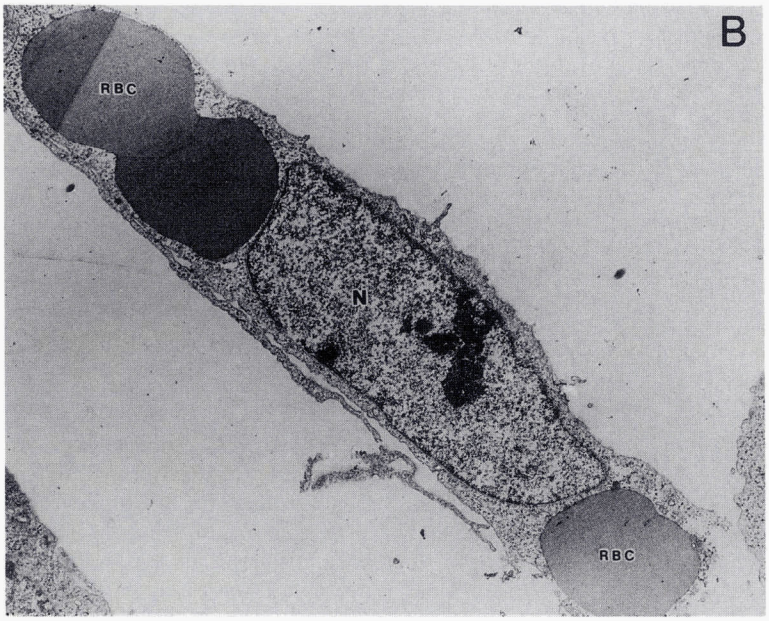

Fig 1. Phagocytosis of IgG-sensitized RBCs by Fcγ receptor transfected COS-1 cells. (A) Light micrograph of a single COS-1 cell transfected with FcγRIIIA and its γ subunit demonstrating the phagocytosis of IgG-sensitized RBCs. (B) Electron micrograph of a COS-1 cell transfected with FcγRIIA is shown. The internalized EA are present within discrete vacuoles. No phagocytosis is observed with sham transfectants or with transfectants incubated with unsensitized RBCs.

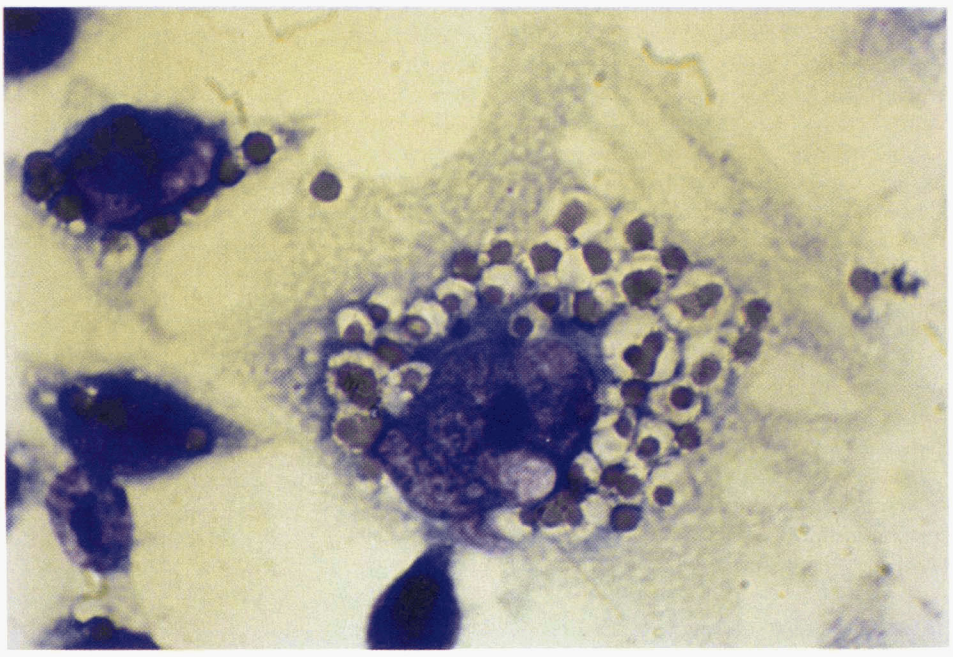

Fig 3. Phagocytosis of IgG-sensitized RBCs by CHO cells transfected with FcγRIIA. Transfection was performed by electroporation of FcγRIIA cDNA inserted into the plasmid PRC/CMV. Stable cell lines of FcγRIIA-expressing CHO cells were established by G418 selection.

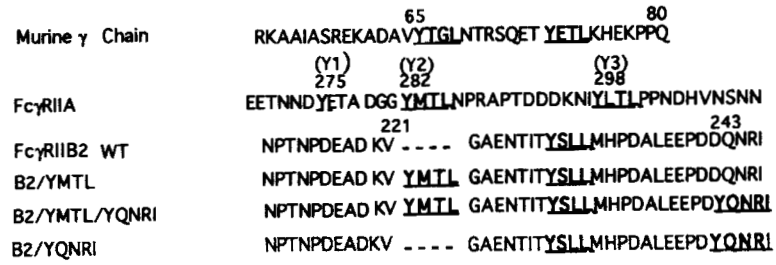

Fig 2. The cytosolic domains of the murine γ chain, FcγRIIA and wild-type and mutant FcγRIIB2 receptors in the region of conserved YXXL sequences. FcγRIIA contains a cytoplasmic region with two YXXL sequences, which is similar to the consensus sequence found in molecules of the Ig gene superfamily implicated in signal transduction. Wild-type human FcγRIIB1 and FcγRIIB2 lack this consensus motif and have only a single cytoplasmic YXXL. YXXL and YXXXI sequences are underlined and in bold face. In the mutant receptor B2/YMTL, the FcγRIIA sequence, YMTL, was introduced into FcγRIIB2 upstream of the existing YSLL. Substitution of aspartic acid 243 with tyrosine created the sequence, YXXXI, and a motif with 13 amino acids separating two of the tyrosines (mutant B2/YMTL/YQNRI). In mutant B2/YQNRI, aspartic acid 243 is replaced by tyrosine in wild-type FcγRIIB2.

tosis has also been shown in transfectants of the mannose receptor, another macrophage cell-surface receptor.[15,17]

We first observed that an isoform of the low-affinity FcγRII receptor FcγRIIA could mediate a phagocytic signal in COS-1 cell transfectants, whereas the high-affinity Fcγ receptor FcγRI could not.[8] FcγRIIIA, which requires its γ subunit for receptor expression,[18] required the γ subunit to induce phagocytosis.[12,13] On the other hand, the FcγRII isoforms, FcγRIIB1 and FcγRIIB2, did not induce phagocytosis.[9] Because these Fcγ 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 Fcγ receptors.

FcγRII

Isoforms of FcγRII arise from the expression of three FcγRII genes, FcγRIIA, FcγRIIB, and FcγRIIC, and alternative splicing of FcγRIIB.[3-7,19] Although the extracellular and transmembrane regions of these isoforms are similar or identical, several of these receptors, eg, FcγRIIA, FcγRIIB1, and FcγRIIB2, show variability in the length and structure of their cytoplasmic domains.[3-7,19] 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.[8,11] Human FcγRIIB1 and FcγRIIB2 contain only a single YXXL sequence in their cytoplasmic domains (Fig 2).[9] Two such YXXL sequences are included in a conserved motif, the ITAM (Ig gene family tyrosine activation motif, D/EX_{2}-YXXL/I$X_{(6-7)}$YXXL/I), observed in the cytoplasmic domains of several Ig gene family receptors such as the T-cell and B-cell receptor systems.[20-30] 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.[8,11] 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,[26] 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 absence of this sequence in the cytoplasmic domain of FcγRIIB1, FcγRIIB2, and FcγRI was responsible for the inability of these receptors to mediate phagocytosis in COS-1 cell transfectants.

Tyrosine phosphorylation of multiple substrates accompanies activation of Fcγ receptors[22,31-33] and is required for Fc receptor mediated phagocytosis in mouse macrophages.[34] Cross-linking of FcγRIIA elicited a strong tyrosine phosphorylation response in COS-1 cells transfected with FcγRIIA.[9,11,22] 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 (MoAb).[9,11] As in mouse macrophages,[34] inhibitors of tyrosine kinases also decreased phagocytosis in COS-1 cell FcγRIIA transfectants.[11]

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.[8,9] 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.[9] Introduction of the membrane proximal YXXL of FcγRIIA, YMTL, into FcγRIIB2 (upstream of the existing YSLL, after the valine at position 221) results in the juxtaposition of two YXXL sequences and the establishment of a motif resembling that found in FcγRIIA (Fig 2).[9] 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.[9] A second mutation in FcγRIIB2 that replaces aspartic acid[243] 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.[9] 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/YNQRI) mediates more efficient phagocytosis of EA.[9] 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 phagocytic competence of the FcγRII family of receptors. Furthermore, FcγRIIB2, which does not mediate phagocytosis, is not readily phosphorylated in EA-activated COS-1 cell transfectants, but B2/YMTL/YQRNI transfectants, which support phagocytosis, are phosphorylated on tyrosine after receptor activation.[9] B2/YMTL transfectants 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.[19,35,36] 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.[11] 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 phagocytosis, 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.[11] 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.[11]

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.[11] 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.[11] 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 phagocytic activity and tyrosine phosphorylation of the receptor.[11] 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,[11,22] 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.[37] 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.[37] Cross-linking of FcγRIIA with EA resulted in tyrosine phosphorylation of the 40-kD band in both cell lines,[37] 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 transfectants of Chinese hamster ovary (CHO) cells express high levels of the receptor and are able to phagocytose large numbers of EA (Fig 3). 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.[38] 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 transfectants are able to mediate immune-complex endocytosis and that this function requires only short stretches of the receptor cytoplasmic domains.[24,39,40] 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.[16] Therefore, there are cytosolic sequences responsible for phagocytosis which are probably distinct from those required for endocytosis.

FcγRIII

The class III Fcγ receptor FcγRIIIA 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.[18,41-45] Association of FcγRIIIA α with the subunits γ and/or ζ occurs through interactions between their transmembrane regions.[46,47] FcγRIIIA/γ is found in macrophages,[18,42,45,48] and both homodimeric and heterodimeric forms of FcγRIIIA occur in natural killer cells that express both the γ and ζ subunits.[42,43] In transfected COS-1 cells, FcγRIIIA 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 FcγRIIIA and for transduction of a phagocytic signal.[12,13] 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 FcγRIIIA.[12,13] Phagocytosis mediated through the γ chain was similarly abolished in mast cells stably transfected with an FcγRIIA/γ chain chimera in which one or both tyrosine residues of the murine γ chain cytoplasmic domain were mutated.[23]

The ζ chain of the T-cell receptor contains sequences homologous to the γ chain, including the conserved YXXL sequences, in its cytoplasmic region. FcγRIIIA also mediates a phagocytic signal through the ζ chain in FcγRIIIA/ζ chain cotransfectants.[12] However, the ζ chain is less than sixfold as efficient in mediating phagocytosis by FcγRIIIA than is the γ chain. This is of interest because phagocytic monocytes/macrophages express FcγRIIIA 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.[49] For example, substituting the internal XX amino acids from the γ chain into the YXXLs of a ζ chain mutant containing the N-terminal pair of YXXL sequences significantly enhanced phagocytosis to values approaching those of wild-type γ.

In vitro kinase assays using lysates of cultured monocytes indicate that the FcγRIIIA γ chain is phosphorylated on tyrosine residues after FcγRIIIA cross-linking.[13] 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.[13] In contrast to the situation with FcγRIIA, 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.[11,13]

Human neutrophils express the FcγRIII receptor, FcγRIIIB. FcγRIIIB is a phosphatidylinositol glycan-linked Fcγ receptor and lacks transmembrane and cytoplasmic domains.[50,51] However, it is capable of participating in transmembrane signaling events such as calcium release, neutrophil degranulation and actin polymerization.[52,53,54] The mechanism(s) by which FcγRIIIB transmits signals is currently being examined. Recent studies have shown that transfected fibroblasts expressing both FcγRIIIB and the type-3 complement receptor (CR3) are able to bind and phagocytose IgG-coated RBCs, whereas cells expressing either CR3 or FcγRIIIB alone are unable to trigger phagocytosis of EA.[55] Recent studies have also shown a close physical relationship between CR3 and FcγRIIIB in neutrophils and transfected fibroblasts,[56,57] and it has been suggested that the lectin-like interactions between CR3 and FcγRIIIB[56,58] activate a pathway to generate phagocytosis in these cells.[55] The mechanism(s) by which such lectin-like interactions stimulate phagocytic signaling by the phosphatidylinositol glycan-linked Fcγ receptor is not yet understood. Other studies suggest that neutrophil FcγRIIIB may stimulate phagocytosis by interacting with FcγRIIA.[52] Such interactions of FcγRIIIB with neutrophil CR3 and/or FcγRIIA may provide additional mechanism(s) through which Fcγ receptors mediate a phagocytic signal.

FcγRI

FcγRI is unique among the Fcγ receptors in being confined to resting cells of a single lineage, the phagocytic monocyte/macrophage.[3-7] COS-1 cell transfectants of FcγRI do not mediate phagocytosis despite avid binding of IgG-coated RBCs.[8,10] However, phagocytosis mediated through FcγRI was observed in monocytes/macrophages that express multiple Fcγ receptors.[10,59,60] Phosphorylation of multiple substrates occurs after cross-linking of FcγRI on monocytes and hematopoietic cell lines,[31-33] and the tyrosine kinase inhibitors tyrphostin 23 and genistein inhibit FcγRI mediated phagocytosis.[10] Thus, despite the absence of tyrosines in the cytoplasmic domain of FcγRI, protein tyrosine phosphorylation is important for FcγRI mediated phagocytosis. These observations further suggested that a cell-specific gene product(s) present in hematopoietic cells such as macrophages but absent in COS-1 cells was required for FcγRI mediated phagocytosis. To investigate this issue, we prepared stable transfectants of human FcγRI in the murine macrophage cell line P388D1. These human FcγRI transfectants were able to phagocytose RBCs specifically targeted to human FcγRI (E-MoAb).[10] Stable transfectants of a mutant FcγRI lacking the cytoplasmic domain also supported the phagocytosis of E-MoAb in murine macrophages, indicating that the cytoplasmic domain of FcγRI was not required for this process.

The requirement for the γ subunit in FcγRIIIA mediated phagocytosis[12,13] suggested that the γ chain might also be an accessory molecule, present in macrophages but absent in COS-1 cells, that is required for FcγRI mediated phagocytic signaling. Coexpression of the γ subunit with either wild-type FcγRI or an FcγRI mutant lacking the cytoplasmic domain permitted FcγRI mediated phagocytosis in COS-1 cells.[10] These results are consistent with observations of FcγRI phagocytic function in murine macrophages and with the finding that FcγRI and the γ chain are associated in monocytes and macrophage-like cell lines.[61-63]

FcγRI 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.[64,65] However, in the absence of its cytoplasmic domain, FcγRI does not transmit a Ca^{2+} signal in COS-1 cell transfectants.[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,[8-14,22,37] the data indicate that their mechanisms for phagocytosis differ. For example, FcγRIIA requires tyrosines within the conserved motif of its own cytoplasmic domain,[11] whereas both FcγRI and FcγRIIIA require the tyrosines within the conserved cytoplasmic region of the γ subunit for phagocytosis.[10,13,66] Furthermore, in contrast to γ chain mediated phagocytosis, some phagocytosis by FcγRIIA is retained in the presence of a single intact YXXL sequence.[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).[8-10] Agents capable of activating protein kinase C such as phorbol esters (eg, phorbol myristate acetate) have been observed to amplify phagocytosis. Phorbol myristate acetate decreases phagocytosis in transfectants expressing FcγRIIIA and the γ chain but modestly increases FcγRIIA mediated phagocytosis.[9,12,67] 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 FcγRI/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 FcγRI/γ.[9-13,66] This observation suggested 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 FcγRIIA mediated phagocytosis.

One possible candidate for this monocyte/macrophage factor was the protein tyrosine kinase Syk.[68] Syk is present in hematopoietic cells[69-73] and coimmunoprecipitates with the γ chain associated with FcγRIIIA in macrophages and with FcϵRI in mast cells.[71-73] Syk is also phosphorylated on tyrosine after cross-linking of FcγRI or FcγRIIIA on cells of the monocyte/macrophage lineage.[48,70-73] Syk dramatically enhanced the phagocytosis of EA by both FcγRIIIA/γ and FcγRI/γ in transfected COS-1 cells (Fig 4).[66] 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.

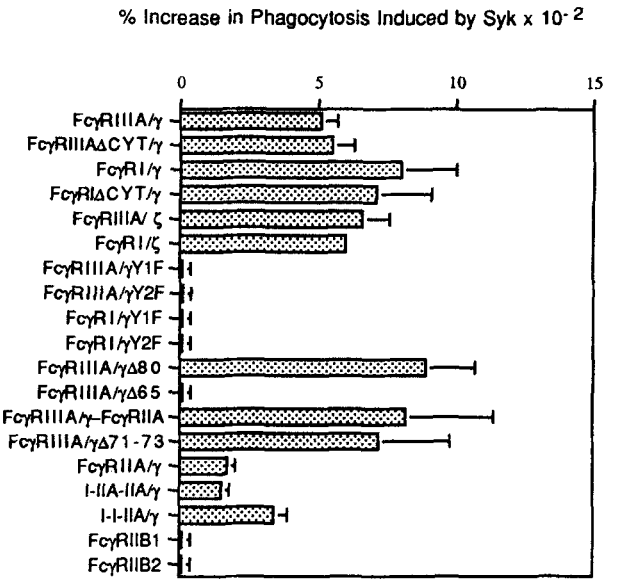

Fig 4. Effect of Syk on phagocytosis mediated by Fcγ receptors. The increase in efficiency of phagocytosis by transfected wild-type and mutant Fcγ receptors in the presence of the PTK Syk is expressed as the percentage of increase in PI (no. of ingested EA/100 Fcγ receptor expressing cells, determined by flow cytometry). Error bars indicate ± SEM. The number of cells that ingest at least one RBC is also increased in the presence of Syk. For example, the fold increase in the percentage of phagocytic cells is 3.0 ± 0.2 for FcγRIIIA/γ, 3.6 ± 0.5 for FcγRI/γ, and 6.3 ± 1.7 for FcγRIΔCYT/γ. FcγRIΔCYT and FcγRIIIAΔCYT indicate mutants in which the cytoplasmic domain has been deleted. γY1F and γY2F indicate γ chain mutants in which either cytoplasmic tyrosine 1 (Y1F) or tyrosine 2 (Y2F) have been replaced with phenylalanine. $\gamma\Delta80$ and $\gamma\Delta65$ are γ chain mutants truncated at amino acid 80 or 65, respectively. $\gamma\Delta71$-73 is a γ chain mutant in which amino acid residues 71-73 have been deleted. The chimeras I-I-IIA and I-IIA-IIA represent receptor molecules containing the EC of FcγRI, the CYT of FcγRIIA, and the TM of either FcγRI or FcγRIIA, respectively. In the γ-FcγRIIA mutant, the sequence between the two YXXLs of the γ chain has been replaced by the sequence between the two YXXLs of FcγRIIA. FcγRI and FcγRIIIA were transfected with the γ chain subunit (eg, FcγRI/γ and FcγRIIIA/γ) or ζ chain as indicated. Data are shown with the γ chain for FcγRIIA and the two FcγRIIA chimeras to show the enhancement by Syk of I-I-IIA mediated phagocytosis in the presence of the γ chain. For most receptors, data are derived from at least four experiments.

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).[66] 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 phosphoryla-

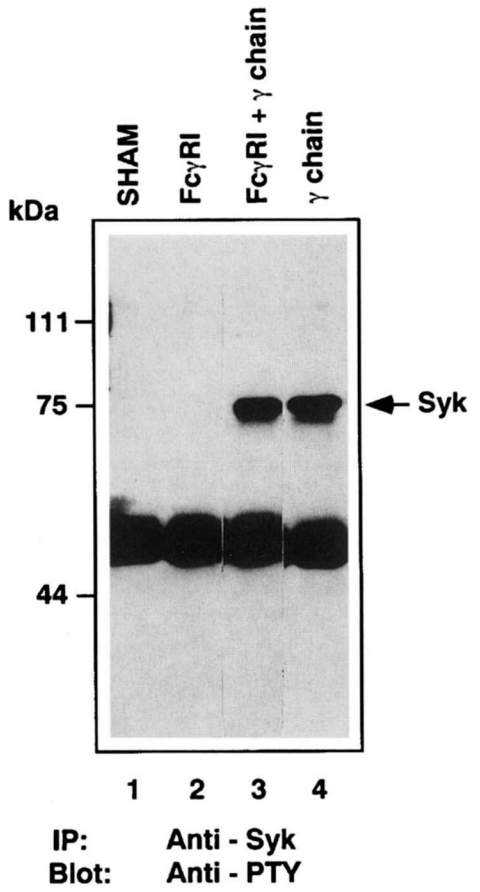

Fig 5. Antiphosphotyrosine immunoblots of stable Syk kinase COS-1 cell transfectants. COS-1/Syk stable transfectants were transfected with FcγRI alone (lane 2), with FcγRI and the γ chain (lane 3), or with the γ chain alone (lane 4). Lane 1 presents the profile of sham transfectants. After incubation with EA, cells were lysed and immunoprecipitated with anti-Syk antibody. In the presence of the γ chain, Syk kinase is phosphorylated on tyrosine.

tion 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 Fcγ-receptor cross-linking enhances tyrosine phosphorylation of Syk kinase.[32,33,48,70,71] 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 γ chain, with or without expression of FcγRI (Fig 5). When COS-1/Syk cells were transfected with γ chain alone or with FcγRI and the γ chain, phosphorylation of Syk was intense, whereas, in COS-1/Syk cells transfected with FcγRI alone, Syk phosphorylation was not observed. This effect occurred in the absence of receptor cross-linking (Fig 5). Thus, γ chain expression alone appears sufficient to induce phosphorylation of Syk in these COS-1 cell transfectants. These data suggest that Syk and the γ chain are associated endogeneously, leading to Syk phosphorylation. Furthermore, taken together,[66,71] our data suggest that receptor cross-linking leads to an alteration in the γ chain/Syk complex that induces Syk kinase activation.

Coexpression of Syk with the ζ chain and either FcγRI or FcγRIIIA also enhances ζ chain mediated phagocytosis but does not increase the level of phagocytosis to that of the γ chain (Fig 4).[66] This result is consistent with previous observations that indicate that the ζ chain is less efficient than the γ chain in inducing phagocytosis in transfected COS-1 cells.[12]

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

In an additional γ chain mutant (γ-FcγRIIA) the sequence separating the two cytoplasmic YXXLs of the γ chain was replaced by the 12 amino acid-intervening sequence from FcγRIIA. This lengthened the sequence between the two γ chain YXXLs from 7 to 12 amino acids, thus creating a γ 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 γ 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 γ chain YXXL sequences allows Syk to function in Fcγ receptor/γ chain mediated phagocytosis.

The induction of Syk phosphorylation after cross-linking of FcγRII in monocytes/macrophages[32,70] suggested that Syk may also play a role in phagocytosis by FcγRIIA. Syk modestly increased the efficiency of phagocytosis by FcγRIIA 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 FcγRIIA and the extracellular domain (EC) of FcγRI (Fig 4).[66]

The use of chimeric receptors also showed the different effects of Syk on FcγRIIA and γ chain induced phagocytosis. Association of the γ chain with FcγRIIIA occurs through the TM of FcγRIIIA.[46,47] Greater enhancement of phagocytosis by Syk in the presence of the γ chain was observed for the chimera I-I-IIA than for either FcγRIIA or the chimeric receptor I-IIA-IIA (Fig 4),[66] which is consistent with the thesis that association of FcγRI with the γ chain also occurs through its TM.[61-63] It is likely that association with the γ chain occurs through the FcγRI derived TM of I-I-IIA and that recruitment of the γ 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-

cytic cells such as monocytes/macrophages.[22,32,33,70,71,75] In contrast to the effect of Syk, cotransfection of the Src family tyrosine kinases (SRTKs) Fgr, Fyn, Lyn, Lck, or Src did not increase FcγRIIIA/γ or FcγRI/γ phagocytosis.[66,76] Nor did these SRTKs further increase the level of phagocytosis induced by Syk.[76] The observation that of these protein kinases, only Syk kinase enhanced phagocytosis by FcγRI or by FcγRIIIA in the presence of the γ chain suggests a specificity of Syk for γ-chain sequences. The low levels of FcγRI/γ and FcγRIIIA/γ mediated phagocytosis in COS-1 cells[10,12,13,66] in the absence of transfected Syk may be due to endogeneous 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 endogeneous 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.[77] In contrast to Syk, the related kinase ZAP-70 did not stimulate FcγRIIIA mediated phagocytosis in transfected COS-1 cells.[49] However, ZAP-70 increased phagocytosis when coexpressed with the Src kinase Fyn.[49] 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 FcγRIII or the T-cell receptor initiates activation of protein tyrosine kinases and induction of a PI-3 kinase-dependent pathway.[78-80] We studied the effect of wortmannin, a specific inhibitor of PI-3 kinase,[81,82] on Fcγ receptor mediated phagocytosis. In COS-1 cell transfectants, wortmannin inhibited Fcγ 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.[81-83] This effect was observed for FcγRIIA mediated phagocytosis as well as for γ chain dependent phagocytosis mediated through FcγRI/γ and FcγRIIIA/γ and was comparable for each Fcγ receptor (Fig 6B). The IC_{50} for inhibition of Fcγ 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.[83,84] In contrast, concentrations of bisindolyl-maleimide, which completely inhibit the activity of protein kinase C,[83] did not inhibit Fcγ receptor mediated phagocytosis (data not shown). These observations indicate that PI-3 kinase is essential for phagocytosis mediated by each of the phagocytic Fcγ receptors.

We have also examined the relationship of PI-3 kinase and Syk in Fcγ receptor transfectants. A phosphorylated band of ≈72 kD that comigrates with Syk is immunoprecipitated by anti–PI-3 kinase from lysates of cross-linked FcγRI transfectants coexpressing FcγRI, the γ chain, and Syk (Fig 7). In addition, after Fcγ 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,[85] 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.

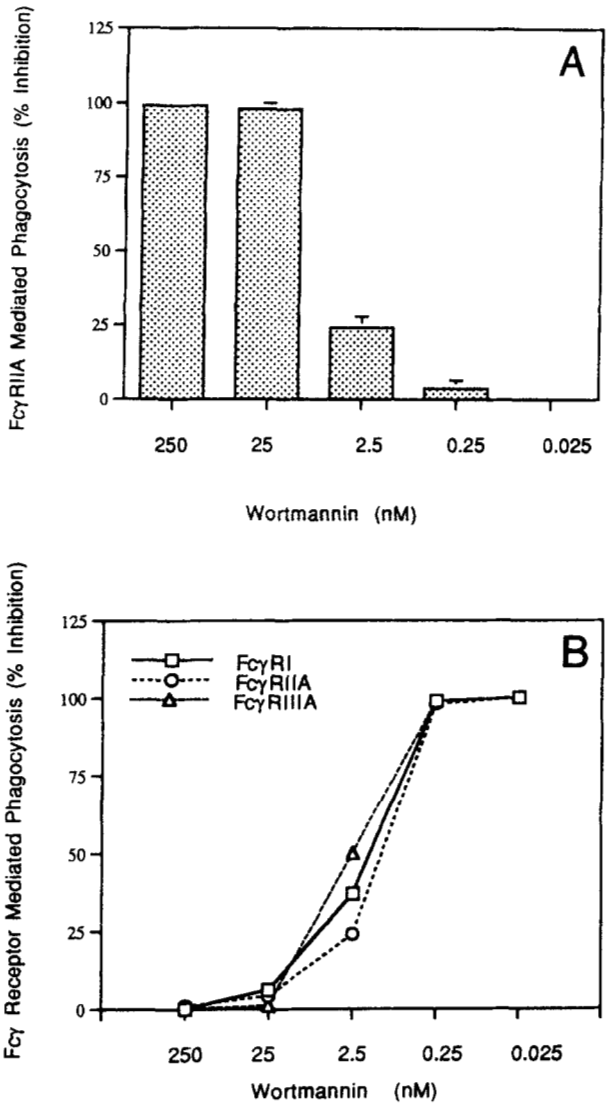

Fig 6. Inhibition of Fcγ-receptor–mediated phagocytosis by the PI-3 kinase inhibitor wortmannin. COS-1 cells were transfected with FcγRIIA, FcγRIIIA/γ, or FcγRI/γ and were examined for phagocytosis of IgG-sensitized RBCs in the presence of wortmannin (0.025 to 250 nmol/L). (A) Inhibition of phagocytosis mediated by FcγRIIA transfectants is shown. In a representative experiment, the PI for the untreated FcγRII transfectant control was 386. In the presence of wortmannin, the PI was 354 (0.25 nmol/L wortmannin), 266 (2.5 nmol/L wortmannin), 11 (25 nmol/L wortmannin), and 0 (250 nmol/L wortmannin). (B) Inhibition of phagocytosis by each phagocytic Fcγ receptor (FcγRI/γ, FcγRIIIA/γ, or FcγRIIA). Inhibition was dose-dependent and was virtually 100% at concentrations of wortmannin that completely inhibit PI-3 kinase activity (25 nmol/L).

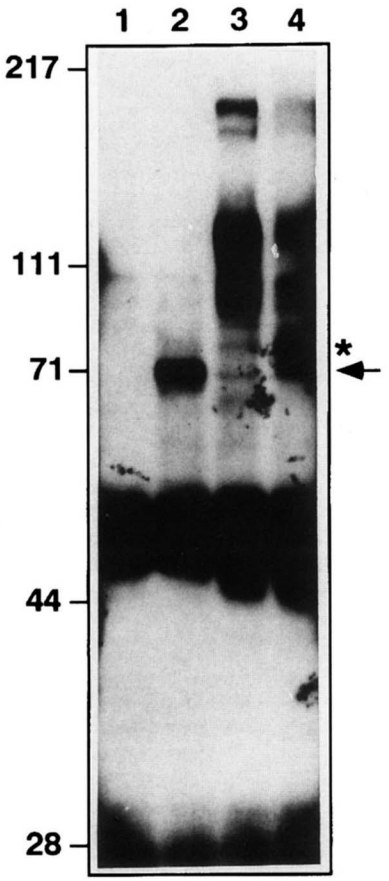

Fig 7. Antiphosphotyrosine immunoblots of FcγRI/γ/Syk COS-1 cell transfectants. Lanes 1 and 3 are sham transfectants, and lanes 2 and 4 are COS-1 cells transfected with FcγRI, the γ chain subunit, and Syk. Lanes 1 and 2, anti-Syk immunoprecipitates; lanes 3 and 4, anti–PI-3 kinase immunoprecipitates. 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.

In summary, isoforms of each class of Fcγ receptor are able to induce the phagocytosis of IgG-coated cells.[8-14,37,59,60] Furthermore, phagocytic function may be induced in certain nonphagocytic cells by the addition of appropriate Fcγ receptors.[8-14,37,38] 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,[8-11] whereas both FcγRI and FcγRIIIA require the tyrosines within the conserved cytoplasmic region of their γ subunit for phagocytosis.[10,13,23] The protein tyrosine kinase Syk markedly enhances the phagocytic signal in the two Fcγ receptors associated with the γ chain.[66] 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.[8-11,22] 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 endogeneously, 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.

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The molecular dissection of Fc gamma receptor mediated phagocytosis

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