Receptors for the Fc domain of IgG on cells of hematopoietic lineage perform important functions, including stimulation of the ingestion of IgG-coated cells. In examining the function of Fcγ receptor isoforms by transfection into COS-1 cells, we have observed that FcγRIIA induces the binding and phagocytosis of IgG-sensitized RBCs (EA) and that transfected COS-1 cells can serve as a model for examining the molecular structures involved in mediating a phagocytic signal. We now report that COS-1 cell transfectants expressing the isoforms FcγRIIB1 and FcγRIIB2 and a FcγRIIA mutant without a cytoplasmic tail efficiently bind EA but do not mediate their phagocytosis. Furthermore, wild-type FcγRIIA, but not FcγRIIB1 or FcγRIIB2, was phosphorylated on tyrosine upon receptor activation. Tyrphostin 23, which alters tyrosine kinase activity, inhibited the phagocytosis of EA and reduced the phosphorylation of FcγRIIA on tyrosine.

Receptors for the constant region of IgG are expressed on the surface of hematopoietic cells and mediate signaling events essential for the effective function of the host defense system. Three classes of Fcγ receptors have been described: FcγRI, FcγRII, and FcγRIII. Within the human FcγRII class, several distinct isoforms have been identified. The diversity is caused by three genes and alternative splicing mechanisms that lead to different gene products whose functions are not well defined. Although the extracellular and transmembrane regions of these isoforms are similar or identical, three of the receptors, FcγRIIA, FcγRIIB1, and FcγRIIB2, exhibit variability in the length and structure of their cytoplasmic domains. The cytosolic tail of FcγRIIA differs both in sequence and length from the cytosolic regions of FcγRIIB1 and FcγRIIB2. FcγRIIB1 and FcγRIIB2 differ from each other by virtue of a 19 amino acid in-frame insertion in the cytosolic tail of FcγRIIB1 caused by alternative splicing. In addition, these isoforms differ in their cellular distribution.

We have recently observed that FcγRIIA expressed in transfected COS-1 cells can efficiently phagocytose IgG-sensitized red blood cells (RBCs) and that activation of FcγRIIB1 and FcγRIIB2 contain one copy of the cytoplasmic sequence YXXL/I implicated in signal transduction, whereas FcγRIIA contains two copies. We therefore inserted YXXL/I sequences at different sites in FcγRIIB2. Low levels of phagocytosis were observed in a FcγRIIB2 mutant bearing the FcγRIIA sequence YMTL and higher levels of phagocytosis were observed in a second FcγRIIB2 mutant that contained both the upstream YMTL and an additional downstream tyrosine-containing motif. Activation of this mutant receptor also induced receptor tyrosine phosphorylation. Thus, these studies indicate that both the number and placement of YXXL sequences in the cytoplasmic domain of the FcγRII receptor family affect both receptor tyrosine phosphorylation and phagocytic competence.

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MATERIALS AND METHODS

Culture and transfection. COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEEM) containing glucose (4.5 mg/mL), glutamine (25 mg/mL), streptomycin (100 U/mL), penicillin (100 μg/mL), and 10% heat-inactivated fetal calf serum. Cells at 70% to 80% confluence were transfected with full-length human Fcγ receptor cDNA in the SV40-based vector pKC4 obtained from Dr Mark Hogarth (University of Melbourne, Melbourne, Australia). Full-length human FcγRIIA cDNA was provided by Dr Mark Hogarth and full-length human FcγRIIB1 and FcγRIIB2 cDNA were provided by Dr Jeffrey Ravetch (Sloan Kettering Institute, New York, NY). Transient transfection of COS-1 cells was performed in complete media containing 10% Nu-Sum (Collaborative Research, Bedford, MA), diethyl aminoethyl (DEAE)-Dextran (1 mg/mL), chloroquine chloride (100 μmol/L), and 2.5 μg plasmid DNA per
milliliter of transfection media. After 4 hours at 37°C, the transfection media was replaced with 10% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS) for 2 minutes at room temperature. The cells were then washed, overlaid with fresh media for further incubation, and analyzed after 48 hours.

**Flow cytometry.** Cell samples incubated with anti-FcγRII MoAb (IV.3) for 30 minutes at 4°C were washed, labeled with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Tago, Inc, Burlingame, CA) for 30 minutes at 4°C, washed and then fixed with 4% paraformaldehyde. Isotype controls were used for all reactions, and fluorescence was measured on a FACSTAR cytometer (Becton Dickinson, Mountain View, CA).

**Binding and phagocytosis of IgG-sensitized RBCs.** Sheep RBCs were sensitized with rabbit antiserum to sheep RBC antibody as previously described. RBCs (Rockland, Gilbertsville, PA) were sensitized by incubation with an equal volume of the highest subagglutinating concentration of rabbit antiserum to sheep RBC antibody (Cappel Laboratories, West Chester, PA) at 37°C for 1 hour. COS-1 cells were incubated with washed EA at 37°C for 30 minutes. Unbound EA were removed by washing and the plates stained with Wright-Giemsa. The percentage of cells binding RBCs was determined by counting in a blinded fashion those cells binding 5 or more sensitized RBCs. To assess phagocytosis, parallel groups of cells were briefly exposed to a hypotonic solution to remove adherent EA. The cells were then stained with Wright-Giemsa and the number of COS cells with one or more internalized EA determined in a blinded fashion.

**Immunoprecipitation and analysis of phosphorylated proteins.** After the removal of externally bound RBCs by brief hypotonic shock, the cells were lysed on plates with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 158 mM NaCl, 10 mM Tris, pH 7.2, 5 mM NaEDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate) at 4°C for 30 minutes. Phosphorylated proteins were immunoprecipitated from lysed cells with affinity-purified rabbit anti-phosphotyrosine antibody UP-28. The immunoprecipitates were analyzed on SDS 7.5% polyacrylamide gels and probed on immunoblots with antiphosphotyrosine monoclonal antibody (MoAb) 4G10. A total of 2 × 10⁶ cells were analyzed per lane. For inhibition studies, cells were pretreated with tyrphostin 23 (50 to 200 μM/L) for 20 minutes at 37°C or with genistein (10 to 25 μg/mL) for 10 minutes at room temperature. Tyrphostin 23 was dissolved in ethanol to a concentration of 10 mM/L. This stock solution was then diluted into 1 mL of PBS to final concentrations of 50, 100, and 200 μM/L of tyrphostin 23, which therefore contained 5, 10, and 20 μM of ethanol, respectively. Genistein (10 mg/mL stock) was also dissolved in ethanol and added into 1 mL of PBS. The final concentrations of genistein were 10 μg/mL and 25 μg/mL. Both the 10 μg/mL and the 25 μg/mL genistein solutions contained 10 μL of ethanol.

**Biotinylation of receptor proteins.** Transfected cells were washed once with PBS, overlaid with 1 mL PBS containing 100 μM/L NaHCO₃ and 100 μM/L Biotin (Pierce, Rockford, IL), and incubated at room temperature for 1 hour. NLCI to 100 μM/L was added and incubation was continued for an additional 10 minutes. The cells were washed once and lysed with RIPA buffer at 4°C for 20 minutes. FcγRIIA was immunoprecipitated with anti-FcγRII MoAb IV.3 and FcγRIIB was immunoprecipitated with anti-FcγRII-specific MoAb 4H11. The immunoprecipitates were analyzed on an SDS 7.5% polyacrylamide gel and the immunoblot was probed with avidin horse radish peroxidase (BioRad, Richmond, CA; 1:1,000 dilution) followed by Enhanced Chemiluminescence reagents (Amersham Corp, Arlington Heights, IL) and visualized using Kodak XAR-5 film (Eastman Kodak, Rochester, NY).

**RESULTS AND DISCUSSION**

To examine differences in phagocytic function among the members of the FcγRII receptor family, we transfected COS-1 cells with FcγRIIA, FcγRIIB1, or FcγRIIB2. Analysis by flow cytometry demonstrated a significant and similar shift in mean fluorescence intensity for these transfectants, indicating that each of these gene products was efficiently expressed on the cell surface (Fig 1). FcγRIIA, FcγRIIB1, and FcγRIIB2 expressed in COS-1 cells also bound IgG-sensitized RBCs (EA) efficiently (Fig 2). No binding was observed in cultures of sham-transfected cells or in transfected cells incubated with unsensitized RBCs. To examine the ability of these isoforms to phagocytose IgG-sensitized RBCs, the externally bound RBCs were removed by hypotonic lysis. In COS-1 cells transfected with FcγRIIA, phagocytosis of sensitized RBCs was detected in 40% to 50% of the cells with bound RBCs (Fig 2). However, even though cells transfected with FcγRIIB1 and FcγRIIB2 bound large numbers of IgG-coated RBCs externally, these cells were unable to phagocytose highly sensitized EA (Figs 2 and 3).

We and others have demonstrated phagocytosis of EA in COS-1 cells and other cell lines derived from fibroblasts after Fcγ receptor transfection. As shown in light microscope studies, the ingested EA are enclosed in distinctive vesicles and some RBCs show partial degradation. There is no phagocytosis in cells transfected with mutant receptors lacking the cytoplasmic domain, although the binding of EA remains unchanged (see below). Cytochalasin D and cold temperature inhibit the ingestion of EA without changing EA binding. In addition, electron micrographs of internalized EA confirm that the COS cell FcγRIIA transfecants mediate phagocytosis that appears morphologically similar to that in monocytes/macrophages.

The functional differences between the FcγRII isoforms most likely arise from differences in structure of the cytoplasmic domain of the FcγRII receptor family (Fig 5). FcγRIIB1 and FcγRIIB2 may lack the essential determinants or, alternatively, carry an element(s) that inhibits the phagocytic process. Transfected COS-1 cells expressing a mutant FcγRIIA receptor lacking the cytoplasmic domain bound EA (25% to 35% of transfectants), but did not mediate phagocytosis of EA (0% of transfectants). Thus, the cytoplasmic domain of FcγRIIA contains determinants needed for phagocytosis of IgG-coated RBCs. This observation is consistent with the experiments of Odin et al., who showed that murine macrophage transfectants expressing a truncated FcγRIIA did not phagocytose EA. The sequence (D/E-X-7-
D/E-X2-Y-X2-L-X6.7-Y-X2-L) is conserved in the cytoplasmic domains of some Ig gene superfamily receptor molecules (or their associated functional subunits) involved in signaling. The cytoplasmic domain of FcγRIIA contains a similar motif with two YXXL conserved sequences, whereas human FcγRIIB1 and FcγRIIB2 contain only a single YXXL.

In COS-1 cells transfected with FcγRIIA cDNA, FcγRIIA is the predominant phosphotyrosine-containing protein induced by EA stimulation. In contrast, cross-linking of FcγRIIA in platelets or HEL cells and monocytes (not shown) induces tyrosine phosphorylation of multiple proteins, including FcγRIIA. The detection of FcγRIIA as the major protein phosphorylated on tyrosine in transfected COS-1 cells may be caused by the absence of substrates that are phosphorylated by the activated tyrosine kinases in platelets, HEL cells, and monocytes, or by the inability of the substrates to couple with kinases in COS-1 cells. Alternatively, in COS-1 cells the phosphorylation of substrates other than FcγRIIA may be below the level of detection because all of the transfected cells do not express FcγRIIA.

To further examine the role of tyrosine phosphorylation in FcγRIIA-mediated phagocytosis, we pretreated the transfected COS cells with inhibitors of tyrosine kinases. Preincubation of FcγRIIA-transfected COS-1 cells with tyrphostin 23 or with genestein caused a dose-dependent inhibition of phagocytosis by FcγRIIA transfectants (Table 1) but no change in the binding of EA. In parallel samples, tyrphostin 23 and genestein caused a significant reduction in tyrosine phosphorylation of FcγRIIA (Fig 6). Although the ethanol buffer alone also inhibited tyrosine phosphorylation, tyrphostin 23 and genestein produced a larger inhibition of tyrosine phosphorylation than an equivalent amount of ethanol buffer. At 100 μmol/L tyrphostin, a concentration that produces a profound reduction in phagocytic index (Table 1), there was also inhibition of tyrosine phosphorylation (Fig 6). The inability of phagocytosis to proceed in the presence of detectable tyrosine phosphorylation of FcγRIIA suggests that tyrosine phosphorylation of substrates other than FcγRIIA may be important for phagocytosis. Thus, these experiments raise the possibility that tyrosine phosphorylation of FcγRIIA and perhaps other substrates plays a role in the induction of phagocytosis by FcγRIIA.

Agents capable of activating protein kinase C (PKC) such as phorbol esters (eg, PMA) have been observed to amplify phagocytosis, including that mediated by Fc receptors. In addition, PMA can impart phagocytic function to some cell surface receptors, such as the complement receptors CR1 and CR3.
(C3b receptor) and CR3 (iC3b receptor), which bind C3-coated RBCs without stimulating phagocytosis. Because we observed that FcγRIIB1 and FcγRIIB2 bind EA without inducing a phagocytic signal, we explored whether PMA can induce these receptors to mediate phagocytosis. PMA did not induce phagocytosis by FcγRIIB1- or FcγRIIB2-transfected COS-1 cells, even though it amplified phagocytosis by FcγRIIA transfectants 2.1-±0.2-fold. This effect occurred in the absence of an increase in Fcγ receptor surface expression as measured by flow cytometry. Thus, stimulation of a phagocytic signal by a PKC-mediated pathway also depends on sequences present in the cytoplasmic domain of FcγRIIA but absent from the cytosolic domains of FcγRIIB1 and FcγRIIB2.

We performed a series of experiments to determine why the FcγRIIB isoforms are not phosphorylated on tyrosine and why they do not induce phagocytosis in COS cell transfectants. Because the murine homologue of human FcγRIIB2 supports phagocytosis of IgG-sensitized T. gondii in transfected cells and contains a cytoplasmic region con-
Fig 4. Electron micrographs of COS cells transfected with FcγRIIA. (A) Binding of EA to transfectants; the arrow indicates an internalized EA. (B and C) Internalization of EA (N, nucleus in Fig 3C). No phagocytosis was observed with sham transfectants or with FcγRIIA transfectants incubated with unsensitized RBCs.
taining two YXXL like sequences, we hypothesized that the addition of YXXL sequences into the cytoplasmic domain of human FcyRIIB2 might provide the necessary environment for phagocytosis by FcyRIIB2. We therefore inserted additional YXXL sequences at different sites in FcyRIIB2 (Table 2). Our studies indicate that the membrane proximal YXXL of FcyRIIA (YMTL) contributes to the phagocytic response by FcyRIIA.33 Therefore, we introduced this YMTL sequence into FcyRIIB2, upstream of the existing YSLL (after the valine at position 221, Fig 5), resulting in the juxtaposition of two YXXL sequences and the establishment of a motif resembling that found in FcyRIIA as well as in many other Ig gene superfamily receptors and/or their subunits.89 This mutation (M1) did not result in full receptor-mediated phagocytic activity but did allow a low, reproducible level of phagocytosis in FcyRIIB2 transfectants (M1 FcyRIIB2 = 0.5% to 2% phagocytic cells, P < .02; wild-type FcyRIIB2 = <0.2% phagocytic cells; and wild-type FcyRIIA = 25% to 40% phagocytic cells) in seven separate experiments and using M1 DNA prepared from three different PCR generated clones. Some of the mutant FcyRIIB2 transfectants contained 5 to 7 ingested RBCs. Because sham and WT (wild-type) FcyRIIB2 transfectants did not phagocytose EA, this small phagocytic signal by M1 FcyRIIB2 is considered significant.

The low phagocytic activity by this FcyRIIB2 mutant indicated that this YXXL replacement is not sufficient for efficient transmission of a phagocytic signal by FcyRIIB2. Therefore, we constructed two additional FcyRIIB2 mutants (Fig 5). Replacement of aspartic acid 243 by tyrosine 11 amino acids downstream of YSLL in M1 creates the sequence, YXXXI, and a motif with 13 amino acids separating two of the tyrosines (mutant M2). In mutant M3, aspartic acid 243 is replaced by tyrosine in WT FcyRIIB2.

**Table 1. Effect of Inhibition of Protein Tyrosine Kinases on Phagocytosis**

<table>
<thead>
<tr>
<th>Tyrosinase 23 (μmol/L)</th>
<th>PI</th>
<th>Phagocytosis of EA (% cells)</th>
<th>Binding of EA (% cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>370 ± 66</td>
<td>23 ± 12</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>50</td>
<td>84 ± 12</td>
<td>10 ± 2</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>100</td>
<td>63 ± 21</td>
<td>7 ± 2</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>200</td>
<td>6 ± 5</td>
<td>3 ± 1</td>
<td>36 ± 1</td>
</tr>
<tr>
<td>Sham</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

COS-1 cells transfected with FcyRIIA were preincubated with the tyrosine kinase inhibitors tyrphostin 23 and genestein17-30 and the binding and phagocytosis was of EA determined. Genestein (10 μg/mL and 25 μg/mL) also reduced phagocytosis of EA by greater than 30%. Tyrphostin and genestein were dissolved in ethanol (10 μL ethanol in 100 μmol/L tyrphostin or 10 μg/mL of genestein). Ethanol alone (10 μL) reduced the phagocytic index 30% to 40%.

Abbreviation: PI, phagocytic index, the average number of ingested RBCs per 100 FcyRIIA expressing COS-1 cell transfectants; tyrphostin 23 inhibited FcyRIIA-mediated phagocytosis in 4 of 4 experiments.
To study this further, we examined the phosphorylation on tyrosine of FcγRIIB2 mutants in transfected COS cells after receptor stimulation with EA (Fig 6). In contrast to the absence of tyrosine phosphorylation in WT FcγRIIB2, the M2 FcγRIIB2 transfectants were phosphorylated on tyrosine after receptor activation (Fig 7, lanes 5 and 6). To a lesser extent, the M1 FcγRIIB2 transfectant was also phosphorylated (Fig 7, lane 4). The FcγRIIB2 mutants were similarly expressed in the COS transfectants, as indicated by the immunoprecipitation studies after their biotinylation on the COS cell surface (Fig 8, lanes 2 and 3). The variation in the migration rates on gel electrophoresis of FcγRIIA and FcγRIIB2 may be caused by differences in glycosylation and is similar to that which has been reported by others.20,21

It has been suggested that in several members of the Ig gene superfamly the amino acids separating the two tyrosines within the YXXL motif assume a helical configuration bringing the tyrosines into apposition.5 In FcγRIIA, the two YXXL tyrosines are separated by 15 amino acids, whereas, in FcγRIIB2 mutant M1, the tyrosines are separated by 10 amino acids. Although the FcγRII three-dimensional structure has not been defined, this suggests a different helical configuration for FcγRIIA and M1 FcγRIIB2. A more nearly FcγRIIA-like configuration might be achieved by M2 FcγRIIB2, since the number of amino acids separating the two downstream motif tyrosines are more similar for M2 FcγRIIB2 and FcγRIIA.

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 do main.34,35 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.36 Therefore, there are cytosolic sequences responsible for phagocytosis that are probably distinct from those required for endocytosis. As with phagocytosis in hematopoietic cells, ingestion of EA by our COS-cell transfectants requires an intact cytoskeleton, as indicated by sensitivity to cytochalasin D.5

The tissue distribution of the human FcγRII subclasses also appears to reflect the functional differences demonstrated here. FcγRIIB1 and FcγRIIB2, which do not mediate phagocytosis, are expressed in lymphoid cells as well as in other cells of myeloid origin.33 In contrast, phagocytosis-competent FcγRIIA is expressed in monocytes, neutrophils, and myeloid cell lines such as U937 and HEL cells, but is absent from B cells or cell lines of B-lymphoid origin not normally associated with Fcγ receptor-mediated phagocytosis.3 These data suggest that FcγRIIB1 and FcγRIIB2 may
function primarily in lymphocytes to mediate activation of
gene programs that do not involve phagocytosis. However,
FcγRIIA, in cells of the phagocytic monocyte/macrophage/
granulocyte lineage, functions as a phagocytic receptor.

In summary, these studies demonstrate the importance of
the cytoplasmic domain in tyrosine phosphorylation and in
phagocytosis of IgG-sensitized cells by FcγRIII. FcγRIIA is
distinguished from the FcγRIIB isoforms by its ability to
induce its phosphorylation on tyrosine and by its ability to
mediate a phagocytic signal. Whether the FcγRIIB isoforms
are able to transmit signals other than phagocytosis is a
subject of future study. Furthermore, these studies implicate
sequences within the cytoplasmic domain of FcγRIIA,
including a double YXXL/I motif, in events important for
phagocytosis and in the induction of tyrosine phosphoryla-
tion of the FcγRII family of receptors. Further studies ana-
lyzing the importance of the tyrosine and leucine residues
within this motif, and the interactions of this motif with
cellular tyrosine kinases, are in progress.

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