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Research Article

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In the Absence of Other Fc Receptors, Fc γ RIIIA Transmits a Phagocytic Signal That Requires the Cytoplasmic Domain of Its γ Subunit

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Abstract

The transmembrane isoform of Fc γ RIII, Fc γ RIIIA, is found on NK cells, cultured monocytes, and tissue macrophages in association with a dimer of an accessory subunit, either γ or ζ . Functions of individual Fc receptors have been difficult to analyze due to coexpression of the receptors on hematopoietic cells and permanent cell lines expressing Fc receptors. cDNAs for the α and γ subunits of Fc γ RIIIA were cotransfected into COS-1 cells, which lack endogenous Fc receptors, to evaluate receptor-mediated phagocytosis and changes in [Ca²⁺]_i. Transfectants both bound and phagocytosed IgG-sensitized erythrocytes and, following activation of Fc γ RIIIA, increased [Ca²⁺]_i. The γ subunit was essential both for the surface expression of the receptor and for transduction of the phagocytic signal. Truncation of the γ subunit cytoplasmic domain (amino acids 65–80) eliminated phagocytic function. Phorbol ester inhibited phagocytosis in a concentration-dependent manner, but did not affect IgG-sensitized erythrocytes binding, suggesting that a protein kinase C-dependent pathway inhibits phagocytosis. The data indicate that a tyrosine containing cytoplasmic domain within the γ subunit is required for phagocytosis by Fc γ RIIIA. (*J. Clin. Invest.* 1993. 92:1967–1973.) Key words: receptors • phagocytosis • signaling • immunoglobulin • macrophages

Introduction

Receptors for the Fc portion of IgG are expressed on many cells of hematopoietic lineage. These receptors are important in host defense and in the ingestion of IgG-coated cells (1, 2). There are three classes of human Fc γ receptors (Fc γ RI, Fc γ RII, and Fc γ RIII), distinguished by IgG subclass affinity, cellular distribution, and reactivity with monoclonal antibodies (1). The genes for these receptors have homology in the coding regions for their extracellular domains, but are divergent in their transmembrane and cytoplasmic regions, suggesting functional specificity for the individual receptors. Fc γ RIII (CD16), a low affinity Fc receptor, has two isoforms that are encoded by two highly homologous genes (3, 4). Fc γ RIIIB is found only on neutrophils as a glycosyl phosphatidylinositol-linked protein (5). Fc γ RIIIA is found on NK cells, cultured monocytes and macrophages (6, 7). The receptor is a multichain complex composed of a ligand-binding polypeptide, α , and a disulfide-linked dimer of an accessory subunit, γ or ζ . The γ and ζ subunits

were originally identified as components of other multimeric receptors, the high affinity receptor for IgE (Fc ϵ RI)¹ and the T cell receptor (TCR/CD3), respectively (8–10).

Examination of the functions ascribed to the individual Fc γ receptors and their signal transduction pathways has proven difficult due to coexpression of more than one class of receptor on hematopoietic cells that express Fc receptors. Further complexity is added by various isoforms of Fc γ RIIIA. The Fc γ RIIIA- α polypeptide associates with a dimer of $\gamma\gamma$, $\zeta\zeta$, or $\gamma\zeta$ that likely interacts with different second messenger pathways and subserve different functions upon immune complex binding (1).

A previous report indicated that the fibroblast-derived COS-1 cell line possessed the cellular machinery for phagocytic cell function following Fc receptor (11) and mannose receptor transfection (12). We used COS-1 cells to evaluate the phagocytic function of the Fc γ RIIIA complex, composed of an α and a homodimer of γ . We observed that COS-1 transfectants phagocytosed IgG-sensitized cells and that the cytoplasmic domain of its γ subunit is required. In addition, we undertook studies with a known inducer of protein kinase C (PKC), PMA, to begin to dissect the signaling pathway involved in phagocytosis by this Fc γ RIIIA isoform.

Methods

Cell culture. COS-1 cells were maintained in DMEM–high glucose (4.5 mg/ml), glutamine (25 mg/ml), penicillin (100 U/ml), streptomycin (100 μ g/ml) and 10% heat-inactivated FCS at 37°C with 5% CO₂. Cells were seeded onto 3.5-cm plates or six-well plates (Falcon Labware, Oxnard, CA) at a concentration of 3×10^5 cells/well, 24 h before transfection. The cells reached approximately 80% confluence for transfection. All tissue culture reagents were obtained from the University of Pennsylvania Tissue Culture Facility.

Construction of recombinant plasmids. The coding sequence of the Fc γ RIIIA- α cDNA (kindly provided by Dr. Bice Perussia, Jefferson College of Medicine, Philadelphia, PA) was ligated into the SV40-driven eukaryotic expression vector pSVL (Pharmacia LKB, Piscataway, NJ) digested with XbaI and SacI. The cDNA for the murine Fc ϵ RI γ -subunit (kindly provided by Dr. Jean-Pierre Kinet, National Institutes of Health, Bethesda, MD) was expressed from the same vector. Truncation mutants of the γ subunit were constructed by using the two-step overlap-extension PCR (13). GAMdel-1, a mutant with the truncation at Lys⁸⁰, was generated by changing the codon AAG for Lys to TAG for a termination. GAMdel-2, a mutant with the truncation at Tyr⁶⁵, was generated by changing the codon TAC for Tyr to TAG for a termination.

Transient transfection of COS-1 cells. Cotransfections of cDNAs were carried out with a modified DEAE-dextran method. Briefly, 300,000 COS-1 cells were seeded on 35-mm well plates, 24 h before transfection. Plates of 70–80% confluency were washed twice and incubated for 40 min with DMEM (GIBCO BRL, Grand Island, NY) with

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1. Abbreviations used in this paper: ADCC, antibody-dependent cellular cytotoxicity; EA, IgG opsonized sheep erythrocyte; Fc γ R, Fc receptor for IgG; Fc ϵ R, Fc receptor for IgE; PI, phagocytic index; PKC, protein kinase C; RBC, red blood cells.

out serum before transfection. 4 μg of plasmid DNA (0.5 $\mu\text{g}/\mu\text{l}$) was slowly added to 1 ml of a transfection buffer containing Nu medium (DMEM with 10% NuSerum [Collaborative Biomedical, Bedford, MA]), 1 mg/ml of DEAE-dextran and 100 μM chloroquine. The transfection buffer containing DNA was added to COS-1 cells with incubation for 4 h at 37°C. Cells were then shocked with 10% DMSO in PBS for 2 min, washed twice with DMEM, and grown in Nu medium. Cells were studied 48 h following transfection.

Antibodies. Anti-Fc γ RIII monoclonal antibody 3G8 (14) was isolated from ascites fluid (the 3G8 hybridoma cell line was kindly provided by Dr. J. Unkeless, Mt. Sinai Medical School, New York, NY) using protein A (Affi-Gel Protein A Maps II; Bio-Rad Laboratories, Richmond, CA). The ascites fluid (1.5 ml) was applied to a 5-ml protein A agarose column. The column was washed with 15 vol of binding buffer, and the IgG was eluted according to manufacturer's instructions. The eluted protein was dialyzed against PBS and concentrated to 7 mg/ml.

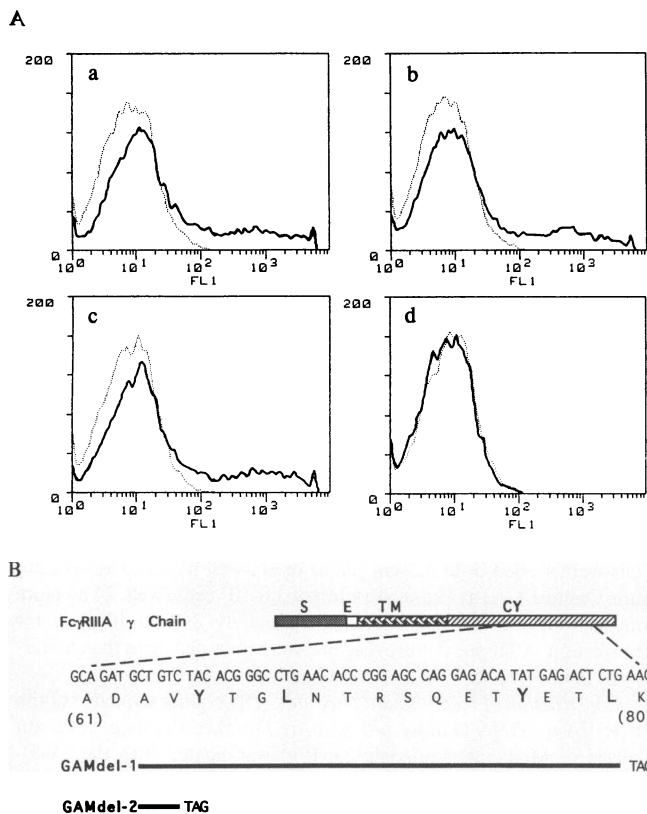


Figure 1. (A) Fluorescence histograms of COS-1 cells expressing Fc γ RIII α - α and - γ (wild type and mutants). COS-1 cells were transfected with Fc γ RIII α - α and - γ cDNAs and assayed 48 h later. The dotted lines represent cells stained with an IgG isotype control P3, while the solid lines represent cells stained with anti-Fc γ RIII α mAb 3G8. Transfection with: (a) Fc γ RIII α α and γ wild type, (b) Fc γ RIII α α and GAMdel-1, (c) Fc γ RIII α α and GAMdel-2, and (d) Fc γ RIII α - α and pSVL vector DNA without γ insert are shown. (B) Schematic representation of γ wild type and mutants. Shown with the schematic diagram of the γ chain are signal sequence (S), external peptides (E), transmembrane domain (TM), and cytoplasmic domain (CY). The expanded area shows an area of the nucleotide sequence of the γ chain containing a conserved motif. The conserved amino acids are denoted by the bold face. The two thick horizontal bars shown at the bottom of the figure represent γ mutants with a termination codon at respective 3' termini but 5' ends of the horizontal bars do not represent a termination (their 5' ends are identical to that of wild type γ).

Table 1. Fc γ RIII α Expression and Phagocytosis by COS-1 Cells Transfected with Fc γ RIII α - α and - γ (Wild Type or Mutants)

Fc γ RIII α	MFI*	PI [§]	Phagocytosis		Rosetting
			% Cells +	% Cells +	
α + pSVL (Sham)	15	0	0		0.61
α + γ (Wild Type)	254	30 \pm 7.1	8.8 \pm 1.5		36 \pm 3.0
α + γ (GAMdel-1)	259	25 \pm 3.5	6.1 \pm 0.7		35 \pm 1.7
α + γ (GAMdel-2)	303	0	0		37 \pm 5.2

COS-1 cells (7×10^5 cells/well) transfected with Fc γ RIII α - α and - γ subunit cDNAs were incubated at 37°C with EA for 40 min. After Wright-Giemsa staining, phagocytosed RBCs were microscopically scored ($\times 1000$). Results are expressed as the mean \pm SEM for phagocytic index, phagocytosis, and EA binding (rosetting). At least three separate experiments were performed for each clone. For each experiment, 300 cells were counted at each of three randomly selected sites. Unsensitized RBCs did not bind to transfectants. * MFI, mean fluorescence intensity; [§] PI, phagocytic index (number of RBCs internalized per 100 COS-1 cells).

Flow cytometry. Anti-Fc γ RIII mAb 3G8 was used to assess protein expression on the cell surface of transfected COS-1 cells. Cells were gently removed from plates, counted, and resuspended in 50 μl staining buffer (1 \times PBS without Ca²⁺/Mg²⁺, containing 0.02% sodium azide, 0.1% BSA) with 5 μl of primary antibody and placed on ice for 45 min. After two washes, FITC-labeled F(ab')₂ goat anti-mouse IgG (GAM) was added and incubation continued at 4°C for 30 min. Isotype controls were used for all reactions. Fluorescence was measured on a FAC-Star[®] (Becton Dickinson & Co., Mountain View, CA). For all samples, 10,000 events were recorded on a log fluorescence scale and mean fluorescence intensity data and contour maps were generated.

Electron microscopy. COS-1 cells were removed from plates by pipetting and washed to remove media. The pelleted specimens were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. After washing three times in 0.1 M cacodylate buffer, pH 7.4, the specimens were fixed in 1% OsO₄ for 30 min at room temperature. Following dehydration in graded alcohol, the specimens were embedded in Epon-EM-Bed-812 (Electron Microscopy Science, Fort Washington, PA). Thin sections were cut using an LKB Ultratome III microtome (Pharmacia Fine Chemicals, Piscataway, NJ). Pictures were taken with a 300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

Preparation of IgG-sensitized red blood cells (EA). Sheep red blood cells (RBC) were sensitized with rabbit anti-sheep RBC IgG as previously described (14). Briefly, 10⁹ sterile sheep RBC (Rockland Inc., Gilbertsville, PA) suspended in 1 ml of calcium- and magnesium-free PBS was sensitized by incubation with an equal volume of PBS containing the highest subagglutinating titer of rabbit anti-sheep RBC antibody (Cappel Laboratories, Cochranville, PA) at 37°C for 30 min. The IgG-sensitized sheep RBC (EA) were washed twice with PBS and resuspended in a final concentration of 10⁹ cells/ml for overlaying on transfected COS-1 cells.

Binding and phagocytosis of IgG-sensitized RBCs. COS-1 cells were incubated with EA at 37°C for 45 min. Unbound EA was removed by extensive washing and stained with Wright-Giemsa to determine the number of cells with bound EA. For the analysis of phagocytosis, COS-1 cells bound with EA (after three washings) were subjected to a brief hypotonic shock (35 s) with hypotonic PBS to remove surface-bound EA before staining. Results were expressed as phagocytic index (PI), the number of intracellular EA/100 COS-1 cells. Phagocytosis was also assessed by electron microscopy (with the assistance of Dr. Steven Douglas, Children's Hospital of Philadelphia, PA). Statistical analysis was carried out by one-way analysis of variance and the Stu-

dent-Newman-Keuls test for comparing means in a multitreatment study using the Primer of Biostatistics (McGraw-Hill, Inc., New York) for the Apple Macintosh Plus.

Effect of PMA on phagocytosis in COS-1 transfectants. PMA (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO. COS-1 transfectants were incubated with increasing concentrations of PMA for 30 min followed by incubation with EA for 40 min. COS-1 cells were washed extensively, extracellular EA removed, and the cells analyzed for internalized EA as described above.

Measurement of $[Ca^{2+}]_i$ following $Fc\gamma RIIIA$ activation. 48 h following transfection with the $Fc\gamma RIIIA$ - α and γ cDNAs, cells adherent to 2.5-cm glass cover slips were taken for single-cell measurement of $[Ca^{2+}]_i$ following cross-linking of $Fc\gamma RIIIA$ (15). Media were aspirated and replaced by HBSS with Fura-2/AM (Molecular Probes, Inc., Eugene, OR). Cells were incubated for 30 min at 37°C, washed, and preincubated for an additional 30 min with biotinylated anti- $Fc\gamma RIII$ mAb 3G8. The cover slip was transferred to a Leidem cell chamber (Medical Systems, Greenvale, NY) and individual cells were analyzed for a rise in $[Ca^{2+}]_i$ following delivery by a micropipette of 50 μ g/ml streptavidin. Fluorescence was imaged with a 100 Fluor objective and a Diaphot epifluorescence microscope (Nikon Inc., Melville, NY). Emission was collected at 520 nm by a photomultiplier tube connected to a Johnson Foundation Spinning Wheel Fluorimeter (University of Pennsylvania Biomedical Instrumentation Group, Philadelphia, PA) and digitally analyzed by an IBM AT clone-based system (Indec Systems, Sunnyvale, CA). In control experiments only streptavidin or biotinylated mAb anti- $Fc\gamma RIII$ was added to the above cells and mock transfectants.

Results

Cell-surface expression of transfected $Fc\gamma RIIIA$. Expression of $Fc\gamma RIIIA$ complexes 48 h after transfection was determined by flow cytometry using anti- $Fc\gamma RIII$ mAb (Fig. 1 A). Surface expression of the $Fc\gamma RIIIA$ - α protein required interaction with the accessory subunit γ , as previously noted (16–18). Two deletion mutants of the γ chain were constructed by introducing stop codons at amino acids 80 (GAMdel-1 mutant) or 65 (GAMdel-2 mutant) (Fig. 1 B). The cDNAs of these two mutants or wild type γ along with the cDNA of α were cotransfected into COS-1 cells and examined for cell-surface expression of transfected receptor complexes. COS-1 transfectants containing wild type or mutant γ exhibited comparable levels of $Fc\gamma RIIIA$ assessed by mean fluorescence intensity and the percentage cells binding EA. Approximately 36% of the COS-1 transfectants containing wild type or mutant γ avidly bound EA (Table I).

Phagocytosis and binding of EA mediated by $Fc\gamma RIIIA$. We examined COS-1 transfectants containing $Fc\gamma RIIIA$ for their ability to bind and phagocytose IgG-sensitized cells. We and others have demonstrated phagocytosis of EA in COS-1 cells and other cell lines derived from fibroblasts, following Fc receptor transfection (11, 19). In this study, we employed electron microscopy to examine phagocytosis in COS-1 cells cotransfected with the $Fc\gamma RIIIA$ α and γ subunits. The thin-section electron micrograph demonstrates that COS-1 cells that lack endogenous expression of Fc receptors are able to phagocytose EA when transfected with $Fc\gamma RIIIA$ (Fig. 2 A). Light microscopic visualization of Wright-Giemsa-stained COS-1 transfectants also revealed phagocytosis of EA. The ingested EA have been found to be enclosed in distinct vesicles, with some RBCs showing partial degradation. Transfectants expressing both the α and γ chains showed a substantial degree of EA binding (\sim 36% of cells) and phagocytosis (\sim 9% of cells)

(Table I, Fig. 2 B). Control transfectants, transfected with $Fc\gamma RIIIA$ - α and pSVL vector without the γ chain cDNA, did not exhibit binding of EA ($<$ 1% of cells).

Internalization of EA was a time-dependent process and increased significantly from 15 to 60 min of incubation. There was approximately a fourfold increase in the phagocytic index at 60 min in comparison to 15 min (data not shown).

γ Chain is essential for phagocytic signal transduction. Although the surface expression of the $Fc\gamma RIIIA$ α chain requires the γ chain, it is not known whether γ is also involved in the signal transduction events required for phagocytosis. To examine its role in transduction of a phagocytic signal, γ wild type or mutants were cotransfected into COS-1 cells along with $Fc\gamma RIIIA$ α . The GAMdel-1 mutant, containing the COOH terminus 7 amino acid deletion, exhibited a 16% decrease in phagocytic activity. However, the GAMdel-2 mutant with the COOH terminus 22 amino acid deletion completely lost its ability to transduce a phagocytic signal (Table I), suggesting that the cytoplasmic region spanning Tyr⁶⁵ to Leu⁷⁹ is critical for the phagocytic signal. These results demonstrate that the γ chain is essential not only for the surface expression of the α chain but also for the phagocytic function of the receptor.

The effect of PMA on phagocytosis. Previous studies have shown that treatment of PMA increases $Fc\gamma RIIA$ mediated phagocytosis in neutrophils and HEL cells (20, 21). In this study, addition of PMA (10^{-6} – 10^{-10} M) progressively decreased phagocytosis in transfectants expressing $Fc\gamma RIIIA$, with more than a fivefold decrease observed at 10^{-7} M. In contrast, transfectants expressing $Fc\gamma RIIA$ exhibited an increase in phagocytic function at lower concentrations (10^{-10} and 10^{-9} M) followed by a modest decrease at higher concentrations (10^{-8} and 10^{-6} M) (Table II). The effect of PMA depended upon its presence during EA incubation and was rapidly reversible. This effect of PMA on phagocytosis suggests that $Fc\gamma RIIIA$ and $Fc\gamma RIIA$ may mediate their phagocytic signals through different pathways. PMA had no effect on the percentage of COS-1 cells binding EA by either receptor.

$[Ca^{2+}]_i$ stimulation following cross-linking of $Fc\gamma RIIIA$. To assess changes in $[Ca^{2+}]_i$, we performed single-cell measurements. Upon cross-linking of $Fc\gamma RIIIA$ with biotinylated anti- $Fc\gamma RIII$ and streptavidin, $[Ca^{2+}]_i$ rose immediately to 2.5- to 7-fold over baseline in 6 out of 24 cells randomly tested (Fig. 3). However, none of 23 mock-transfected cells exhibited an increase of $[Ca^{2+}]_i$ flux when incubated with biotinylated anti- $Fc\gamma RIII$ and streptavidin (data not shown). Streptavidin alone

Table II. PMA Effect on $Fc\gamma R$ -mediated Phagocytosis in COS-1 Transfectants

	Control	10^{-10} M	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M
$Fc\gamma RIIA$	61±13	88±19	80±29	46±6	47±11	22±4
$Fc\gamma RIIIA$	34±10	33±10	20±6	10±3	6±2	1±1

PMA was added to cells 30 min before incubation with EA and was present during EA incubation. EA was allowed to bind to transfectants for 40 min before staining. Results are expressed as the mean $PI \pm SEM$, $n = 5$, $P < 0.05$. For experiments with $Fc\gamma RIIA$, 2 μ g of pSVL containing $Fc\gamma RIIA$ and equal amounts of the vector DNA without insert were transfected into COS-1 cells. For $Fc\gamma RIIIA$, 2 μ g each of pSVL containing cDNAs of α and γ cDNA were used for cotransfection.

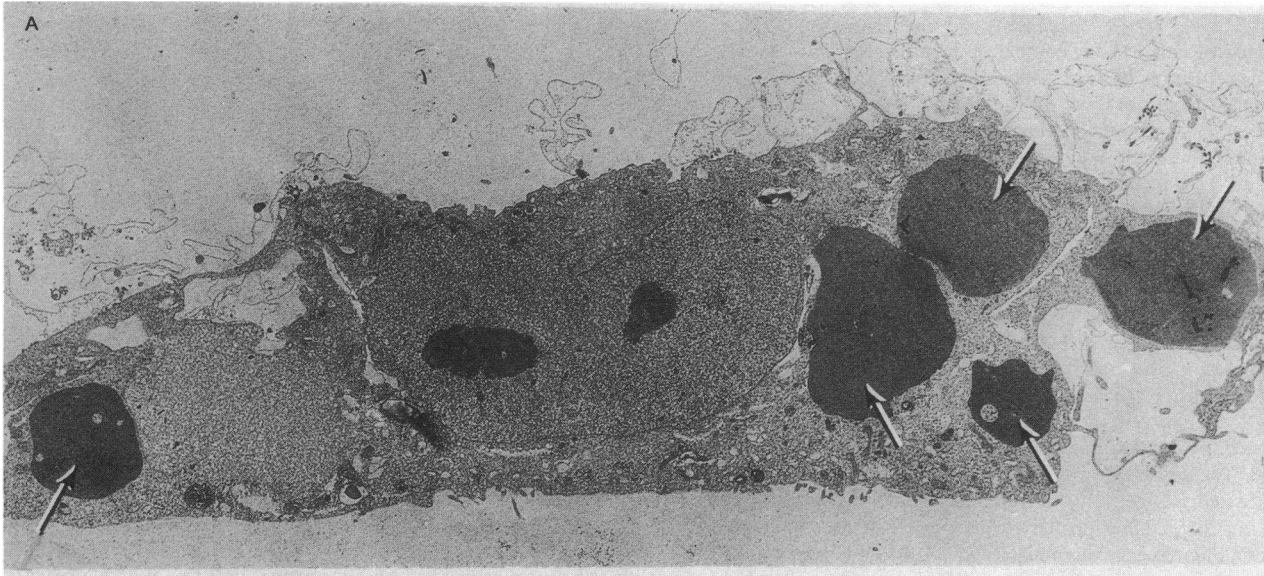


Figure 2. Phagocytosis and EA binding by COS-1 cells expressing Fc γ RIIIA. COS-1 transfectants were incubated with EA for 30 min. Unbound erythrocytes were removed by washing and examined for EA binding. For phagocytic analysis, washed COS-1 cells were treated with a hypotonic solution for 35 s to remove bound EA. (A) Thin-section electron micrograph of COS-1 transfectants demonstrates phagocytosis of EA denoted by arrows. (Picture shows an image magnified by 8000.) (B) The COS-1 cells were stained with Wright-Giemsa and visualized by a light microscope ($\times 1000$). (a) Control cells from mock-transfection incubated with EA. (b) EA binding (rosetting) in COS-1 cells expressing Fc γ RIIIA. (c) Phagocytosis of EA by COS-1 transfectants.

did not stimulate a rise in $[Ca^{2+}]_i$. Epinephrine (10 μ M), which evokes a Ca^{2+} signal in COS cells (22) also stimulated a rise in $[Ca^{2+}]_i$ (Fig. 3).

Discussion

Human Fc γ RIIIA is a receptor complex composed of three separate polypeptide chains, the ligand-binding subunit Fc γ RIIIA- α , and its associated γ and ζ chains found on NK cells, activated monocytes, and macrophages (23). These associated chains belong to a family of polypeptides that dimerize, interact in a noncovalent manner with other polypeptides in multimeric receptors, and are critical for signal transduction by the individual receptors (24, 25). The γ and ζ chains are required for surface receptor expression of Fc ϵ RI and TCR/CD3, respectively (8–10). It has recently been shown that the γ or ζ subunit significantly increases Fc γ RIIIA- α surface expression in cotransfection experiments (16, 18). Without the interaction of the γ or ζ molecule, the α chain is targeted for degradation in the endoplasmic reticulum (23). Fc γ RIIIA can exist in three molecular forms with the α chain associated with either $\gamma\gamma$, $\zeta\zeta$, or $\gamma\zeta$. The Fc γ RIIIA- α - $\gamma\gamma$ isoform is found in macrophages, while all three forms occur in NK cells that express both the γ and ζ subunits (26, 27). We chose to evaluate the role of one specific isoform, Fc γ RIIIA- α - $\gamma\gamma$, in signaling following binding of Fc γ RIIIA by IgG-coated erythrocytes (EA) or anti-Fc γ RIII mAb in a transient transfection system.

Prior studies evaluating individual Fc receptor functions have relied upon the use of cells expressing more than one class of Fc receptor and receptor-specific IgG subclasses or mAbs to simulate ligand binding. Under these conditions, the relative binding specificity, displacement of mAbs by immune complexes, or cooperativity between the cytoplasmic domains of the different Fc receptor classes may influence the results. Fc receptor-negative COS-1 cells have previously been shown to

be an effective transfection system for evaluating mannose receptor (12) and for individual Fc receptor function (11, 19). Thus, analysis of Fc receptor signaling in this system can occur without the potential interpretation that the cytoplasmic domains of different receptors interact following ligand binding. Our results obtained with thin-section electron microscopy further demonstrate that COS-1 cells are an attractive model system to study the phagocytic signal transduction pathway.

COS-1 cells cotransfected with α and γ (wild type and mutants) subunits of Fc γ RIIIA demonstrated comparable levels of cell-surface expression of the multimeric Fc γ RIIIA (Table I). Transfection of cells with Fc γ RIIIA- α chain alone did not induce its cell-surface expression. Thus, our data indicate that the γ chain is necessary for both the surface expression of Fc γ RIIIA and its transmission of a phagocytic signal. During the preparation of our manuscript, it was shown by others that the γ subunit of Fc γ RIIIA plays a critical role in antigen presentation in transformed B cells (28) and in Ca^{2+} signaling in transfected T and mast cells (29). Our results further demonstrate that the γ subunit of Fc γ RIIIA is essential for the signal transduction ability of this receptor. It is of note that the region deleted in the GAMdel-2 mutant contains two conserved tyrosines and leucines present in several signaling molecules of the immunoglobulin gene superfamily (30, 31).

An increase in $[Ca^{2+}]_i$ was demonstrated with COS-1 cells expressing wild type Fc γ RIIIA α and γ following receptor cross-linking, suggesting that the Fc γ RIIIA- α - $\gamma\gamma$ isoform can initiate events involved in Ca^{2+} mobilization. The cytoplasmic domain required for a phagocytic signal, described above, also appears to play an important role for intracellular Ca^{2+} mobilization, as mutants of the γ chain in which tyrosine was replaced by phenylalanine exhibited significant reductions in intracellular Ca^{2+} mobilization (31a). Intracellular Ca^{2+} increases have been demonstrated for all three Fc γ receptor classes (2, 32, 33). Similarly, it has been observed that both

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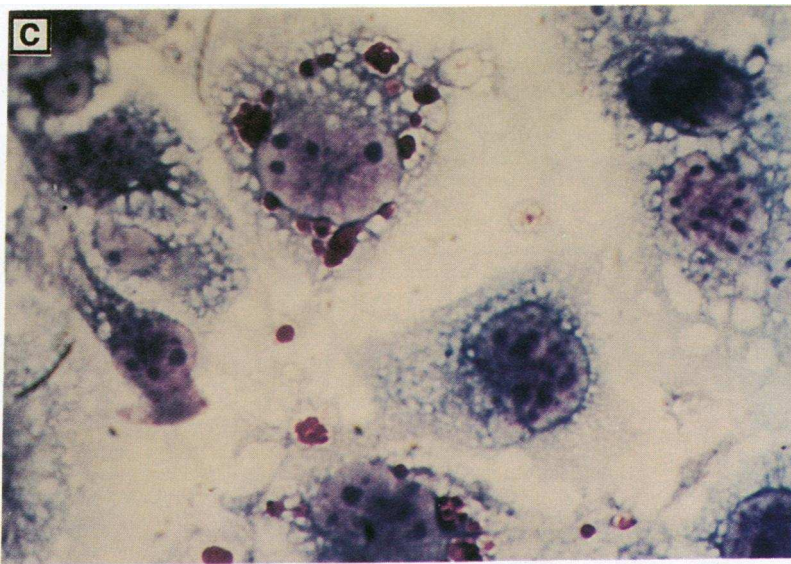
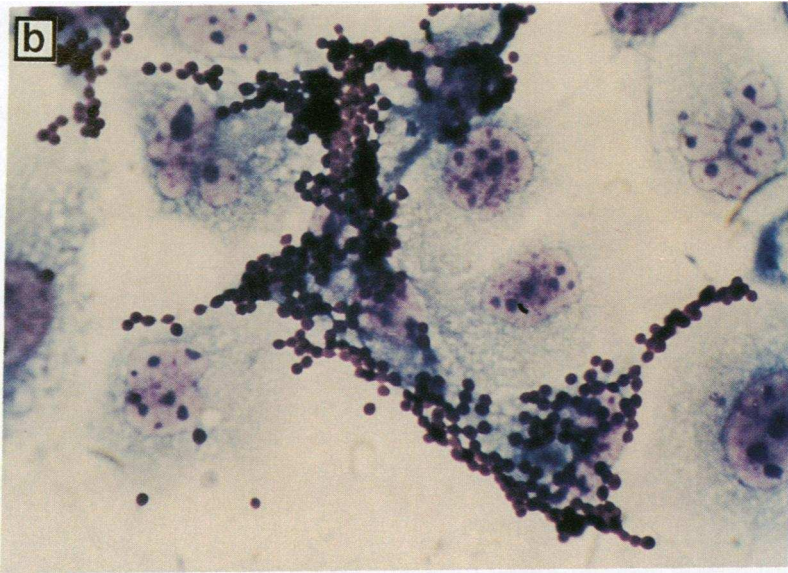
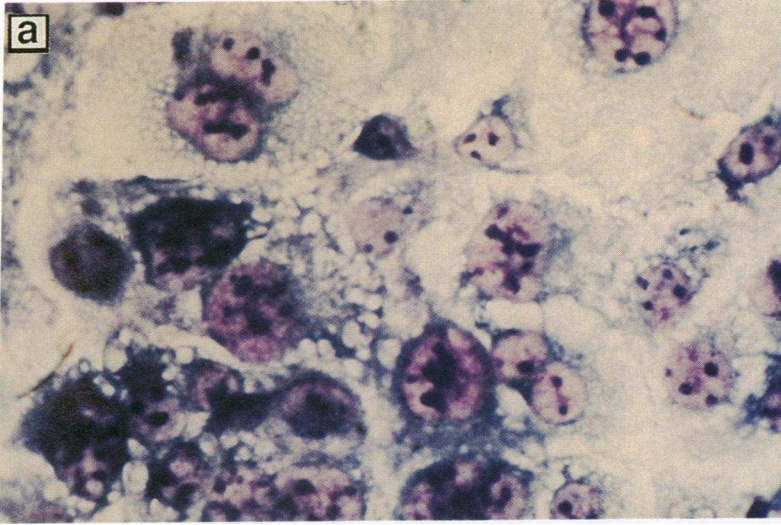


Figure 2 (Continued)

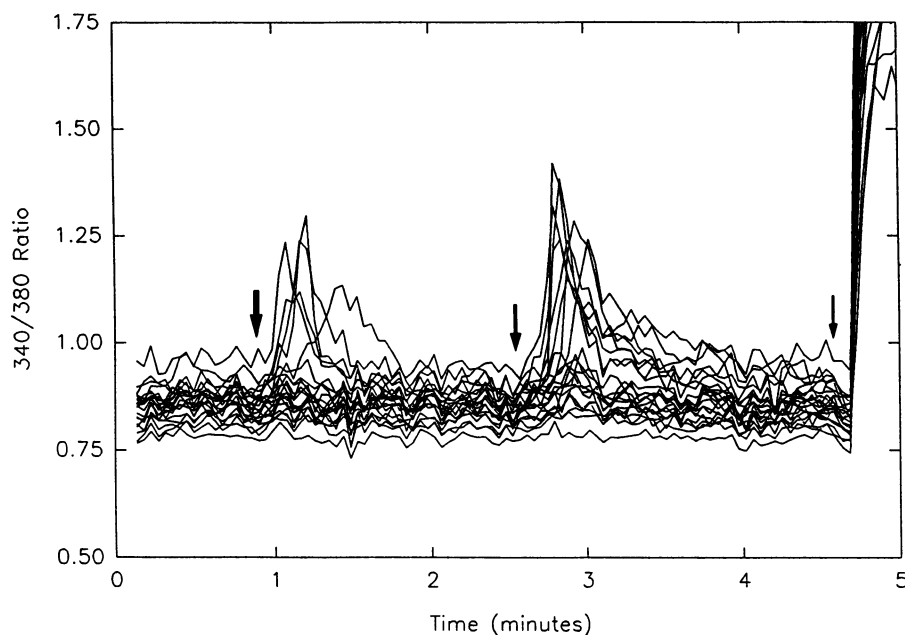


Figure 3. $[Ca^{2+}]_i$ increase on a single cell level following cross-linking of $Fc\gamma RIII A$. COS-1 transfectants plated on glass cover-slips were loaded with Fura-2/AM. After obtaining baseline $[Ca^{2+}]_i$, bound biotinylated anti- $Fc\gamma RIII$ was cross-linked by the addition of streptavidin. In control experiments, only streptavidin or biotinylated anti- $Fc\gamma RIII$ was added with no increase in $[Ca^{2+}]_i$ over baseline. The arrows from left to right (the thickest to the thinnest) represent three time points when streptavidin (following preincubation with biotinylated anti- $Fc\gamma RIII$), epinephrine, and ionomycin are added, respectively.

TCR/CD3 isoforms, $CD3\zeta$ and $CD3\eta$, also transmit signals responsible for Ca^{2+} mobilization (24).

The $Fc\gamma RIII A-\alpha-\gamma\gamma$ isoform mediated both binding and phagocytosis of IgG-sensitized erythrocytes in COS-1 transfectants without the presence of other Fc receptors. Since $Fc\gamma RIII A$ is an abundant $Fc\gamma$ receptor on macrophages and the γ chain is the only member of its family expressed in these cells, these experiments suggest that the $Fc\gamma RIII A-\alpha-\gamma\gamma$ isoform in macrophages plays an important role in phagocytosis. This process may involve increases in intracellular Ca^{2+} (34). In NK cells, different $Fc\gamma RIII A$ signaling pathways involved in ADCC and NK-cell activation may be mediated by $\gamma\zeta$ or $\zeta\zeta$ isoforms.

Through stimulation of the second messenger pathway involving PKC, PMA can affect events in the phagocytic process (20, 21). We evaluated the effects of PMA on phagocytosis in COS-1 transfectants expressing either $Fc\gamma RIII A$ or $Fc\gamma RII A$. PMA (10^{-7} M) has been known to elicit superoxide anion production in U937 cells (35) and to stimulate both complement receptor-mediated phagocytosis in cultured monocytes (36) and $Fc\gamma RII$ -mediated phagocytosis in neutrophils and HEL cells (20, 21). However, the same concentration of PMA (10^{-7} M) caused an 82% reduction of phagocytosis in COS-1 transfectants expressing $Fc\gamma RIII A$, while phagocytosis was reduced only by 13% in $Fc\gamma RII A$ transfected cells. That $Fc\gamma RII A$ transfectants consistently show moderate increases at 10^{-10} – 10^{-9} M of PMA suggests that there are differences between these two $Fc\gamma$ receptors in intracellular signaling. These receptors may mediate phagocytosis in macrophages and stimulate monocytes by coupling with different second messenger pathways. PKC upregulation may be able to stimulate the $Fc\gamma RII$ -mediated pathway, while inhibiting phagocytosis by $Fc\gamma RIII A$.

We have used a model system to study the role of individual Fc receptors, in transmission of a phagocytic signal. We observed that macrophage $Fc\gamma RIII A$ is able to mediate phagocytosis and that the γ subunit of the receptor is essential both for surface expression and for signal transduction mediated by the receptor following activation. Future studies will examine the

role of different isoforms of $Fc\gamma RIII A$ and map the area and individual amino acids critical for transmission of the phagocytic signal.

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