

# Signal Transduction by Fc $\gamma$ RIII (CD16) Is Mediated through the $\gamma$ Chain

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## Summary

To determine the functional role of the two isoforms of Fc $\gamma$ RIII (CD16) (IIIA, IIIB), the signal transduction capabilities of wild-type and mutant forms of these receptors were analyzed in transfected lymphoid, myeloid, and fibroblastic cell lines. Functional reconstitution of receptor signalling was observed in hematopoietic T and mast cells, and was absent in nonhematopoietic (CHO) cells.

Fc $\gamma$ RIIIA, a hetero-oligomeric receptor composed of a ligand-binding subunit  $\alpha$  and dimeric  $\gamma$  chains, generated both proximal and distal responses in Jurkat and P815 cells, typical of what is seen in natural killer cells and macrophages upon receptor activation. In contrast, Fc $\gamma$ RIIIB, which is normally attached to the cell surface via a glycosyl-phosphatidylinositol anchor, was incapable of transducing signals. After crosslinking, Fc $\gamma$ RIIIA signalling was dependent only upon the  $\gamma$  chain. Fc $\gamma$ RIIIA chimeras in which the  $\alpha$  subunit transmembrane and cytoplasmic domains were substituted with the corresponding  $\gamma$  chain sequences functioned as well as wild-type hetero-oligomeric receptors. These data indicate that the ability of the Fc $\gamma$ RIIIA complex to activate the appropriate pathways for cell activation is cell-type restricted and independent of the transmembrane and cytoplasmic domains of the  $\alpha$  subunit. The presence of the  $\gamma$  chain is responsible for the assembly of, as well as the signal transduction by, the functional cell surface complex.

**B**inding of antibodies to effector cells through receptors recognizing their constant regions (FcR) is central to the pathway that leads to clearance of antigens by pinocytosis of immune complexes, phagocytosis of antibody-coated particles, or antibody-dependent cellular cytotoxicity (ADCC)<sup>1</sup> (1). FcRs act as signal-transducing molecules and thus provide an important link in the communication between the innate and the adaptive immune systems. Receptors for the Fc domain of IgG are present on most hematopoietic cells, including B-, T-, and NK cells, macrophages and neutrophils, and most cell types coexpress different forms of FcRs (2).

Three classes of human FcR for IgG (Fc $\gamma$ R) have been defined based upon their different protein and genomic structures: the high-affinity Fc $\gamma$ RI (CD64), and the low-affinity Fc $\gamma$ Rs Fc $\gamma$ RII (CD32) and Fc $\gamma$ RIII (CD16) (3). Evidence suggests that Fc $\gamma$ Rs couple ligand binding to intracellular signalling events, but the contribution of each particular type of Fc $\gamma$ R to the overall cell activation process is still unknown.

Although many early biochemical events such as stimulation of phospholipase C, mobilization of intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>), and activation of protein kinase C are thought to occur after occupancy and crosslinking of these receptors (1-3), the molecules that are responsible for these phenomena have not been unambiguously identified.

To clarify the functional role of FcRs, we have examined the properties of the low-affinity Fc $\gamma$ RIII, which exists in two isoforms. The appearance of these two isoforms is the consequence of a small number of single nucleotide substitutions in the two genes coding for Fc $\gamma$ RIIIA and Fc $\gamma$ RIIIB, leading to either a conventional transmembrane molecule with a distinct cytoplasmic domain, or a glycosyl-phosphatidylinositol (GPI) linked protein, respectively (4-6). Both isoforms have nearly identical extracellular domains, and therefore demonstrate identical ligand-binding specificities. The monomeric GPI-linked Fc $\gamma$ RIIIB is uniquely expressed on neutrophils (7, 8), while the other isoform, Fc $\gamma$ RIIIA, is expressed on NK cells and on macrophages, and exists as a hetero-oligomeric receptor complex consisting of a ligand-binding  $\alpha$  chain associated with disulfide-linked  $\gamma$  or  $\zeta$  chains (9-13). The associated  $\gamma$  or  $\zeta$  chains are required for cell surface expression of Fc $\gamma$ RIIIA $\alpha$ , and are presumed to be involved in signal transduction (9-12). The  $\gamma$  and  $\zeta$  subunits

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<sup>1</sup> *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; GPI, glycosyl-phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PNH, acquired paroxysmal nocturnal hemoglobinuria.

were initially identified as necessary components of the multimeric high-affinity FcR for IgE (FcεRI), and the TCR/CD3 complex, respectively (14, 15).

To explore the capability of the different isoforms of FcγRIII to generate intracellular signalling events, we have introduced the respective genes for FcγRIIIA and FcγRIIIB into the FcR-deficient human leukemic T cell line Jurkat, the murine mast cell line P815, and fibroblastic CHO cells, and subsequently analyzed the capabilities of these receptors to mediate early and late cell activation events elicited by mAbs specific for FcγRIII.

Our data indicate that FcγRIIIB is not able to mediate any of the responses examined. However, the FcγRIIIA complex, upon engagement with mAbs and subsequent cross-linking with secondary antibodies, resulted in responses indistinguishable from the ones initiated by TCR/CD3 activation of Jurkat cells, or FcγRIIIA (CD16) activation of NK cells. A mutant FcγRIIIA complex lacking the cytoplasmic domain of the α chain demonstrated the same activation profile as the wild-type receptor complex. Similarly, a chimeric receptor, composed of the extracellular domain of FcγRIIIAα coupled to the transmembrane and cytoplasmic domains of γ, was sufficient to elicit intracellular responses. These data suggest that the expression of the associated γ chains of FcγRIIIA are necessary to prevent the complex from (ER) endoplasmic reticulum degradation (16) and cell surface expression of the receptor complex (9–11), and are also required in transducing the signal initiated by occupancy of the ligand-binding α chain of the complex.

## Materials and Methods

**Reagents.** Fura-2 acetoxyethyl ester (fura-2/AM) was obtained from Molecular Probes, Inc. (Eugene, OR). Phorbol 12-myristate 13-acetate (PMA), ionomycin, and A23187 were from Calbiochem Corp. (La Jolla, CA). <sup>125</sup>I(100 mCi/ml), myo-[<sup>3</sup>H]-inositol (92 Ci/mmol), [<sup>32</sup>P]orthophosphate (10 mCi/ml), and [<sup>3</sup>H]thymidine (20 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). Geneticin (G418) was obtained from Gibco Laboratories (Grand Island, NY). 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodogen) was purchased from Pierce Chemical Co. (Rockford, IL). Phosphatidylinositol-specific phospholipase C (PI-PLC, isolated from *Bacillus cereus*) was from Boehringer Mannheim Corp. (Indianapolis, IN). All other reagents were from Fluka Chem. Corp. (Ronkonkoma, NY).

**mAbs.** The following antibodies were used: mouse anti-human CD3ε: OKT3 (IgG2a, Ortho Diagnostic Systems, Inc., Raritan, NJ); mouse anti-human CD16: 3G8 (IgG1, prepared in vitro from 3G8 hybridoma (17) grown in a hollow fiber cartridge, Mini Flo-Path; Amicon, Beverly, MA) purified with goat anti-mouse (GαM) IgG-coupled agarose (Sigma Chemical Co., St. Louis, MO); mouse anti-human CD64: 32.2 (IgG1, Medarex, Inc., West Lebanon, NH); mouse anti-human IL2 receptor α chain (IL-2Rα; CD25, IgG1, biotin conjugate; Boehringer Mannheim); mouse anti-phosphotyrosine: 4G10 (IgG2bk; Upstate Biotechnology Inc., Lake Placid, NY); GαM IgG F(ab')<sub>2</sub> free of intact IgG by silver stain analysis of SDS-PAGE analytical gels (Tago Inc., Burlingame, CA).

**Cell Lines.** Jurkat T cells were cultured in medium containing RPMI 1640 supplemented with 10% heat-inactivated FCS, glutamine (2 mM), nonessential amino acids (0.1 mM), penicillin (100

U/ml), streptomycin (100 μg/ml) at 37°C, 5% CO<sub>2</sub>. P815 and CHO cell lines were cultured in the same medium, but RPMI 1640 was replaced by MEM-α and DME, respectively. All cell lines were checked regularly for the presence of mycoplasma and eventually cured with mycoplasma removal agent (ICN Biomedicals, Inc., Costa Mesa, CA).

**FcγRIII Expression Vectors.** cDNA encoding the glycosyl phosphatidylinositol (GPI)-linked form of human FcγRIII(FcγRIIIB) (5) was cloned into the expression vector pNeo (18). cDNAs encoding human FcγRIIIAα and murine FcεRIγ were cloned together with the neomycin resistance gene into the expression vector pcEXV-3 (pcEXV-3N/FcγRIIIAα+γ) (19). A cytoplasmic deletion clone of FcγRIII was described previously (FcγRIIIB with Ser<sub>203</sub> changed to Phe) (11). This mutated cDNA was cloned together with the cDNA encoding the γ chain and the neomycin resistance gene into pcEXV-3 (pcEXV-3N/FcγRIIIAα(Δ)+γ). The chimeric FcγRIIIAα/γ, which contains the extracellular domain of FcγRIIIAα attached directly to the transmembrane and cytoplasmic portions of FcεRIγ, was constructed using PCR methods, and cloned together with the neomycin resistance gene into pcEXV-3 (pcEXV-3N/FcγRIIIα/γ). The resulting plasmids were verified by dideoxynucleotide DNA sequence analysis.

**DNA Transfection.** Jurkat cells were transfected by electroporation, as previously described (20). 48 h after transfection, cultures were adjusted to an active concentration of 0.5 mg/ml G418 sulfate (Geneticin; Gibco Laboratories). After 1 wk, the concentration of G418 was increased to 1 mg/ml. Cultures were fed weekly. Growing clones were detected 3–4 wk after transfection, expanded, and screened for FcγRIII expression by flow cytometry. FcγRIII<sup>+</sup> cells were isolated using the anti-FcγRIII mAb 3G8, and Dynabeads coated with GαM IgG (DynaL Inc., Great Neck, NY). All transfected Jurkat clones were kept in selective medium, and have been phenotypically stable for FcγRIII expression for more than 6 mo of continuous culture. P815 cells and CHO cells were transfected using the calcium-phosphate method, selected in the presence of G418, and cloned as described above.

**Flow Cytometric Analysis.** Cells (10<sup>6</sup>/50 μl) were incubated with 50 μl of antibody (1 μg/ml) for 30 min at 4°C. Cells were washed twice with PBS containing 0.25% BSA (PBS/BSA), and were subsequently incubated with 50 μl of FITC-conjugated GαM IgG F(ab')<sub>2</sub> (1 μg/ml; Tago Inc.). Cells were resuspended in 300 μl PBS/BSA and analyzed using a FACScan® (Becton Dickinson & Co., San Jose, CA).

**Cell-Surface Labeling and Immunoprecipitation.** 10<sup>7</sup> cells were surface-labeled with <sup>125</sup>I by using 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril. Labeled membrane proteins were extracted with lysis buffer containing 1% digitonin and 0.12% Triton X-100. For immunoprecipitation, cell lysates were sequentially incubated (2 h, 4°C for each incubation) with indicated antibodies and with protein A sepharose CL4B or protein A sepharose CL4B coated with RαM IgG antibody (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ), and washed with digitonin buffer four times. Immune complexes were eluted into sample buffer and separated on 10% tricine/SDS-PAGE. Autoradiographic exposures were done at -70°C for 3 d.

**Measurement of [Ca<sup>2+</sup>]<sub>i</sub>.** Measurements of intracellular free calcium levels were performed with fura-2/AM. Cells (5 × 10<sup>6</sup>) were washed once and loaded with 3 μM of fura-2/AM in 500 μl of a 50-mM Hepes buffer (pH 7.2) supplemented with 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 5 mM KCl, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, and 5 mM glucose. After 15 min of incubation at 37°C, the cell suspension was diluted to 10<sup>6</sup> cells/ml with the same buffer, and incubated for an additional 30 min at 37°C. Cells were washed twice

and adjusted to  $10^6$  cells/ml in Hepes buffer. Before analysis, the cells were pre-equilibrated at  $37^\circ\text{C}$  for 10 min. Fluorescence of the stirred cellular suspension was continuously monitored with an Alphascan fluorimeter (Photon Technology International Inc., New Brunswick, NJ).  $[\text{Ca}^{2+}]_i$  was measured and computed as described previously (21).

**Phosphoinositide Hydrolysis.** Jurkat cells or P815 cells were labeled with myo- $[\text{^3H}]$ -inositol ( $10 \mu\text{Ci/ml}$ ,  $10^6$  cells/ml) for 6 h in inositol-free RPMI 1640 supplemented with 10% dialyzed FCS. The cells ( $5 \times 10^6/\text{ml}$ ) were pre-equilibrated at  $37^\circ\text{C}$  and sequentially stimulated with 3G8 ( $1 \mu\text{g/ml}$ ) and  $\text{G}\alpha\text{M}$  IgG F(ab')<sub>2</sub> ( $10 \mu\text{g/ml}$ ) in the presence of 10 mM LiCl. The soluble inositol phosphates were extracted with TCA ( $200 \mu\text{l}$ ) and applied to 1 ml of AG 1-X8 (formate form) 1 ml ion exchange columns (Bio-Rad Laboratories, Richmond, CA) pre-equilibrated with 0.1 M formic acid. After loading of the samples, columns were washed with 10 ml H<sub>2</sub>O and 10 ml 60 mM ammonium formate/5 mM disodium-tetraborate, and elution was performed with increasing concentrations of ammonium formate (0.1–0.7 M) (22). The eluted radioactivity was quantified by liquid scintillation counting in Aquasol (NEN DuPont, Boston, MA).

**Tyrosine Phosphorylation.** For tyrosine phosphorylation analysis,  $2 \times 10^6$  cells were incubated for 3 h at  $37^\circ\text{C}$  in 800  $\mu\text{l}$  phosphate-free DME supplemented with 0.5% dialyzed BSA and glutamine. The cells were labeled with [ $^{32}\text{P}$ ]orthophosphate ( $1 \text{ mCi/ml}$ ) for 4 h at  $37^\circ\text{C}$ , and stimulated with mAbs for 2.5 min, and with or without crosslinking by  $\text{G}\alpha\text{M}$  IgG F(ab')<sub>2</sub> for an additional 2.5 min. Cells were pelleted and resuspended in 300  $\mu\text{l}$  lysis buffer (50 mM Hepes (pH 7.45), 150 mM NaCl, 1% digitonin, 5 mM EGTA, 50  $\mu\text{g/ml}$  leupeptin, 10  $\mu\text{g/ml}$  aprotinin, 2  $\mu\text{g/ml}$  pepstatin, 1 mM PMSF, 200  $\mu\text{M}$  sodium orthovanadate, 20 mM NaF, and 20 mM sodium pyrophosphate), incubated at  $4^\circ\text{C}$  for 1 h, and clarified by centrifugation at 14,000 rpm for 15 min at  $4^\circ\text{C}$ . Immunoprecipitations were carried out from supernatants by addition of 1  $\mu\text{g}$  antiphosphotyrosine mAb 4G10 for 16 h at  $4^\circ\text{C}$ . Protein A sepharose CL4B (Pharmacia LKB Biotechnology, Inc.) was added for 1 h, then the immune complexes were washed three times with lysis buffer and prepared for SDS-PAGE (10%). Autoradiograms were exposed at  $20^\circ\text{C}$  for 16 h. Each lane represents  $\sim 2 \times 10^6$  cells.

**IL-2 Production and IL-2R Expression.** Jurkat cells were incubated with the indicated mAbs for 30 min at  $4^\circ\text{C}$ . After removal of unbound antibody, the cell concentration was adjusted to  $10^5$  cells/ml, and 200  $\mu\text{l}$  aliquots were transferred to 96-well microtiter plates that were precoated with  $\text{G}\alpha\text{M}$  IgG and blocked with RPMI plus 10% FCS. PMA and A23187 were added at final concentrations of 10 ng/ml and 1  $\mu\text{M}$ , respectively. After 36 h of culture, IL-2 activity was assessed by measuring [ $^3\text{H}$ ]thymidine uptake of the IL-2-dependent cell line CTLL in a 1:4 dilution of the test supernatants. Means of triplicate determinations are given. SD were  $<10\%$  of the means. Neither PMA, A23187, nor any of the mAbs used in this study had effects on this assay by themselves.

IL-2R $\alpha$  expression was determined by flow cytometry of stimulated cells using biotin-coupled IL-2R $\alpha$  mAb and FITC-coupled streptavidin (Boehringer Mannheim, Inc.) using a FACScan® (Becton Dickinson & Co.).

## Results

**Expression of Different Isoforms of Fc $\gamma$ RIII on Transfected Jurkat Cell Lines.** The stable expression plasmids for Fc $\gamma$ RIIIB, Fc $\gamma$ RIII $\alpha$ + $\gamma$ , and Fc $\gamma$ RIII $\alpha$ ( $\Delta$ )+ $\gamma$  were linearized by restriction enzyme digestion and introduced into recipient Jurkat T cells using electroporation. Flow cytometry of the

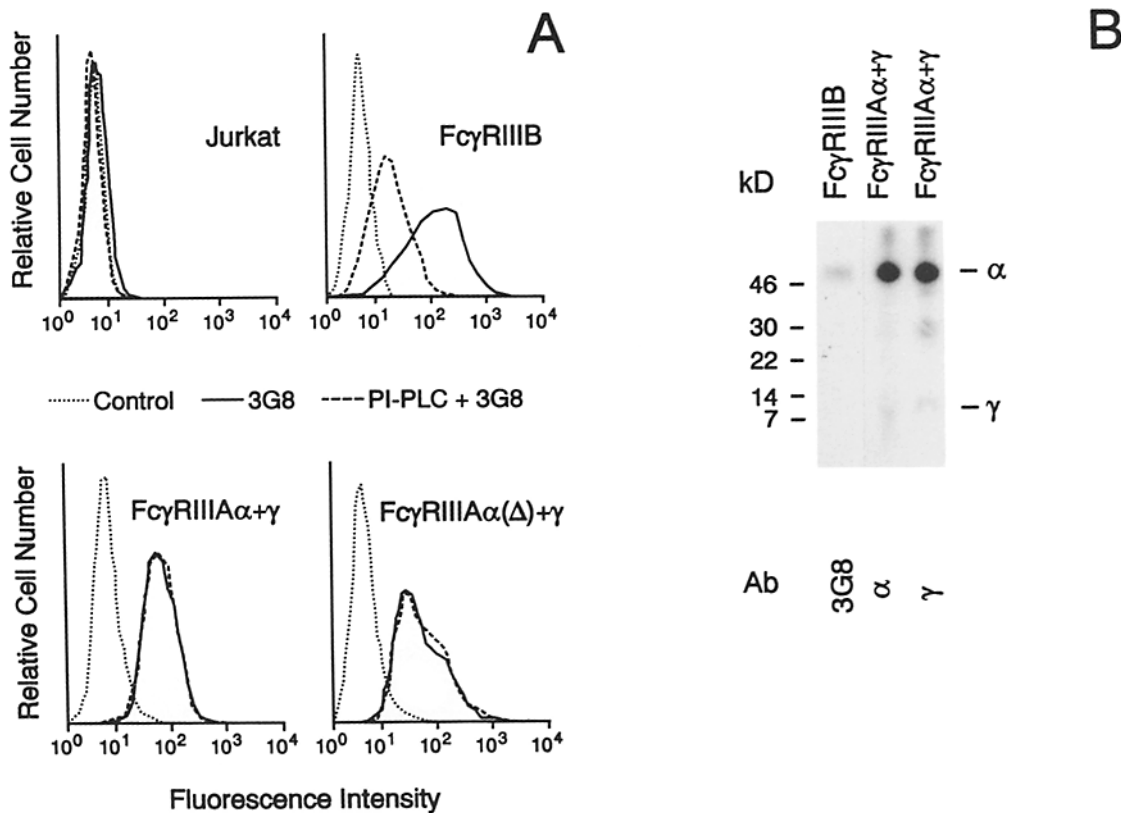
established cell lines showed that the different forms of Fc $\gamma$ RIII were equally expressed on the transfected Jurkat cells (Fig. 1 A). Cell surface expression of Fc $\gamma$ RIII $\alpha$  and Fc $\gamma$ RIII $\alpha$ ( $\Delta$ ) were obtained only by cotransfection with the  $\gamma$  chain. As expected, Fc $\gamma$ RIIIB was susceptible to digestion with PI-PLC, while Fc $\gamma$ RIII $\alpha$ + $\gamma$  and Fc $\gamma$ RIII $\alpha$ ( $\Delta$ )+ $\gamma$  were resistant (Fig. 1 A).

To confirm the flow cytometric data and to determine the subunit composition of the receptor complexes, cells were surface labeled with radioactive iodine and subsequently lysed under mild detergent conditions to preserve receptor complexes. The Fc $\gamma$ RIII isoforms were immunoprecipitated from the lysates using a variety of antibodies (Fig. 1 B). 3G8, a mAb directed against the extracellular domain of Fc $\gamma$ RIII, immunoprecipitated a broad band of protein from cells expressing Fc $\gamma$ RIIIB characteristic of this glycoprotein (Fig. 1 B, 3G8). Polyclonal antiserum raised against the cytoplasmic domain of Fc $\gamma$ RIII $\alpha$  did not react with extracts from the same cells (data not shown).

Immunoprecipitation from extracts of Jurkat cells expressing Fc $\gamma$ RIII $\alpha$ + $\gamma$  with the anticytoplasmic antibody of Fc $\gamma$ RIII $\alpha$  resulted in two major bands: the  $\alpha$  and the  $\gamma$  chains of the receptor complex (Fig. 1 B,  $\alpha$ ). Similarly, when extracts from these cells were reacted with an antiserum against the  $\gamma$  chain, the same bands were observed, confirming the association of the  $\alpha$  and  $\gamma$  chains in the receptor complex (Fig. 1 B,  $\gamma$ ). The faint bands appearing between the  $\alpha$  and  $\gamma$  chains at about 30 kD are likely to represent components of the endogenous TCR/CD3 complex that have been shown to associate with Fc $\epsilon$ RI $\gamma$  (23). Extracts from Jurkat cells expressing the mutant Fc $\gamma$ RIII $\alpha$ ( $\Delta$ )+ $\gamma$  complex reacted only with 3G8 or the  $\gamma$  antiserum (data not shown). None of the immunoprecipitating antibodies reacted with molecules from extracts of nontransfected parental cells (data not shown). Thus, Fc $\gamma$ RIIIB appears not to be associated with any other molecule, while Fc $\gamma$ RIII $\alpha$  is preferentially associated with the cotransfected Fc $\epsilon$ RI $\gamma$ , rather than with the endogenous TCR/CD3 $\zeta$ . As we have found in COS cells (11), complex formation of  $\alpha$  and  $\gamma$  in Jurkat cells is independent of the cytoplasmic domain of Fc $\gamma$ RIII $\alpha$ .

The molecular appearance of the Fc $\gamma$ RIII isoforms in Jurkat cells reflects the situation in vivo, where on neutrophils Fc $\gamma$ RIIIB is expressed as a GPI-linked monomer (4–8), while on NK cells or macrophages Fc $\gamma$ RIII $\alpha$  appears as a receptor complex consisting of one membrane spanning  $\alpha$  chain associated with either a  $\gamma_2$  or  $\zeta_2$  homodimer, or a  $\gamma/\zeta$  heterodimer (9, 12, 13).

**Crosslinking of Fc $\gamma$ RIII $\alpha$  Leads to an Increase in  $[\text{Ca}^{2+}]_i$ .** The Fc $\gamma$ RIII-expressing Jurkat cells allowed us to determine the signalling capacity of these molecules in the absence of other FcRs. We therefore examined the ability of 3G8 to elicit an increase in  $[\text{Ca}^{2+}]_i$  in Jurkat cells transfected with Fc $\gamma$ RIII $\alpha$ + $\gamma$ , Fc $\gamma$ RIII $\alpha$ ( $\Delta$ )+ $\gamma$ , or Fc $\gamma$ RIIIB. Fig. 2 depicts typical fluorometry tracings obtained with the different Jurkat clones upon stimulation of Fc $\gamma$ RIII or TCR/CD3, and subsequent crosslinking of the antibody-coated cell surface molecules with  $\text{G}\alpha\text{M}$  IgG F(ab')<sub>2</sub>. Stimulation of the wild-type Fc $\gamma$ RIII $\alpha$ + $\gamma$  complex and the cytoplasmic deletion clone



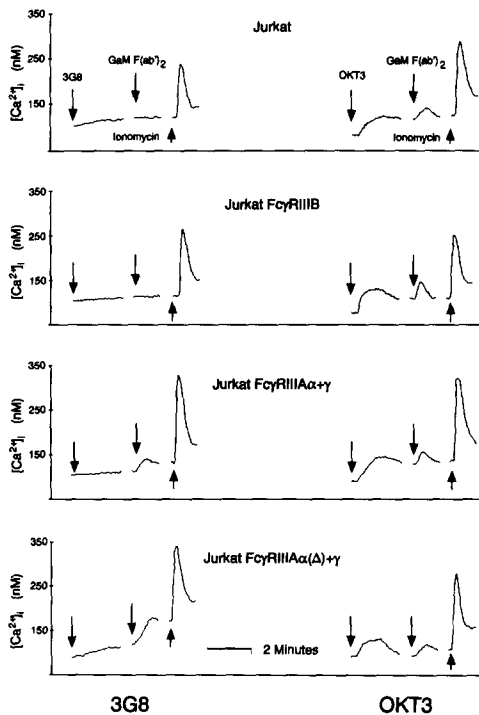
**Figure 1.** (A) Cell surface expression and PI-PLC sensitivity of different isoforms of FcγRIII in transfected T cells. Nontransfected Jurkat cells were used as the control. Indicated cell lines were incubated with 3G8 followed by labeling with fluorescein-conjugated GαM F(ab')<sub>2</sub> (solid lines). Non-specific staining was assessed by using an isotype-matched negative control mAb 32.2 (dotted lines). To assess PI-PLC sensitivity, cells were incubated in PBS/0.5% BSA in the presence of PI-PLC (2.5 U/ml) at 37°C for 90 min before flow cytometric analysis (dashed lines). (B) Immunoprecipitation of surface-iodinated Jurkat cells expressing FcγRIII. Surface radio-iodinated cells were lysed in 1% digitonin lysis buffer and immunoprecipitated with anti-FcγRIII mAb 3G8, antiserum raised against the cytoplasmic portion of FcγRIIIAα (α) or anti-FcεRIγ antiserum (γ). Immunoprecipitates were analyzed under reducing conditions by gel electrophoresis, followed by autoradiography. The weaker signals obtained using 3G8 are due to lesser immunoprecipitation potency of this mAb compared with the polyclonal α and γ antibodies. Overexposure of FcγRIIIB did not show any associated bands. The respective clones and antibodies used are indicated. Each lane represents ~10<sup>7</sup> cells. Positions of the molecular weight standards are indicated (kD).

FcγRIIIAα(Δ)+γ with 3G8 and subsequent crosslinking resulted in transient rises in [Ca<sup>2+</sup>]<sub>i</sub>. The concentration of 3G8 as shown in Fig. 2 was chosen to give an optimal increase after addition of the crosslinking GαM IgG F(ab')<sub>2</sub>. Higher concentrations of 3G8 resulted in a brisker increase in [Ca<sup>2+</sup>]<sub>i</sub>, but with no further response after crosslinking with GαM IgG F(ab')<sub>2</sub> (data not shown). Stimulation of FcγRIIIB with 3G8 and subsequent crosslinking with GαM IgG F(ab')<sub>2</sub> resulted in no detectable increase in [Ca<sup>2+</sup>]<sub>i</sub>. OKT3 was used as a positive control in all cell lines examined.

**Stimulation of FcγRIIIA Results in Activation of PIP<sub>2</sub> Hydrolysis and Tyrosine Kinase Pathways.** Since the increases in [Ca<sup>2+</sup>]<sub>i</sub> that occur with TCR/CD3 stimulation are attributed to increases in inositol phosphates, we tested the ability of FcγRIII to induce PIP<sub>2</sub> hydrolysis by assessing changes in total soluble inositol phosphates after stimulation with 3G8 (Fig. 3A). Stimulation of the FcγRIIIA complex with 3G8 resulted in the generation of inositol phosphates in both the wild-type FcγRIIIAα+γ and the cytoplasmic deletion mutant FcγRIIIAα(Δ)+γ. In contrast, no inositol phosphates were generated after stimulation of FcγRIIIB in transfected cells. Although the initial increase of PIP<sub>2</sub> hydrolysis in cells

expressing FcγRIIIAα+γ and FcγRIIIAα(Δ)+γ was only minor, subsequent crosslinking of the 3G8 coated cells led to a considerable increase in the generation of inositol phosphates. No such increase was observed in nontransfected Jurkat cells (Fig. 3A). These results are consistent with the calcium fluorometry data and confirm the ability of FcγRIIIA to activate PIP<sub>2</sub> hydrolysis.

Stimulation of TCR/CD3 in T cells, FcγRIIIA in NK cells and macrophages, and FcεRI in basophils and mast cells activate tyrosine phosphorylation, in addition to PIP<sub>2</sub> hydrolysis (24–27). We therefore sought to determine if activation via FcγRIII would result in tyrosine kinase activation in our assay system as well. Immunoprecipitation with anti-phosphotyrosine antibodies from <sup>32</sup>P-labeled cell extracts revealed that a small number of tyrosine phosphoproteins were present in all unstimulated cells. (Fig. 3B, Control lane). Stimulation of cells with 3G8 or the crosslinking GαM IgG F(ab')<sub>2</sub> alone had no effect in the different FcγRIII-expressing Jurkat cells. Subsequent crosslinking of 3G8 coated cells with GαM IgG F(ab')<sub>2</sub> resulted in the appearance of a pattern of tyrosine phosphoproteins in cells expressing FcγRIIIAα+γ and FcγRIIIAα(Δ)+γ that was indistinguishable



**Figure 2.** Calcium mobilization by Fc $\gamma$ RIII in transfected Jurkat cells. Nontransfected Jurkat cells, cells transfected with Fc $\gamma$ RIIIB, Fc $\gamma$ RIIIA $\alpha$ + $\gamma$  or Fc $\gamma$ RIIIA $\alpha$ ( $\Delta$ )+ $\gamma$  loaded with fura-2/AM were stimulated with anti-Fc $\gamma$ RIII mAb (3G8; 0.1  $\mu$ g/ml), or anti-CD3 $\epsilon$  mAb (OKT3; 1  $\mu$ g/ml), and subsequently crosslinked with G $\alpha$ M F(ab')<sub>2</sub> (10  $\mu$ g/ml). Ionomycin (1  $\mu$ M) was used as a receptor-independent control. Cells were analyzed for changes in [Ca<sup>2+</sup>]<sub>i</sub> by fluorometry as described in Materials and Methods. (Arrows) Stimuli added.

able from that seen after stimulation with OKT3 and cross-linking. Thus Fc $\gamma$ RIIIA $\alpha$ + $\gamma$  and Fc $\gamma$ RIIIA $\alpha$ ( $\Delta$ )+ $\gamma$  are both able to activate tyrosine phosphorylation in a manner analogous to that of the TCR/CD3 complex. No stimulation was detected in cells expressing Fc $\gamma$ RIIIB and in nontransfected control cells.

**Stimulation of Fc $\gamma$ RIII Results in Late Events of T Cell Activation.** T cell activation results from the transmission of receptor-mediated signals from the cell surface to the nucleus where they act to induce expression of specific genes (28). The expression of the IL-2 gene is tightly regulated, requiring the integration of a number of signals for its transcription, and making it a valuable distal marker for assessing signalling through cell surface receptors.

A significant increase in IL-2 production was found in cells that were sequentially stimulated with OKT3 and crosslinked with immobilized G $\alpha$ M IgG (Fig. 4 A). Stimulation with 3G8 resulted in a comparable increase in IL-2 production, but only in the cells expressing either Fc $\gamma$ RIIIA $\alpha$ + $\gamma$  (Fig. 4 A) or Fc $\gamma$ RIIIA $\alpha$ ( $\Delta$ )+ $\gamma$  (data not shown).

Another gene that is used as an activation marker in T cells encodes the IL-2 receptor  $\alpha$  chain (IL-2R $\alpha$ , CD25) (28). Although the level of IL-2R expression after stimulation with 3G8 is lower than with OKT3, the pattern of induction is similar to the one observed for IL-2 production (Fig. 4 B). These data demonstrate that in addition to early signal trans-

duction events, late activation events occur upon stimulation of Fc $\gamma$ RIIIA, but not Fc $\gamma$ RIIIB, thus demonstrating its ability to couple to the relevant signal transduction pathways in a physiologic manner. The cytoplasmic domain of Fc $\gamma$ RIIIA $\alpha$  subunit is not necessary for either the early or the late activation events examined here.

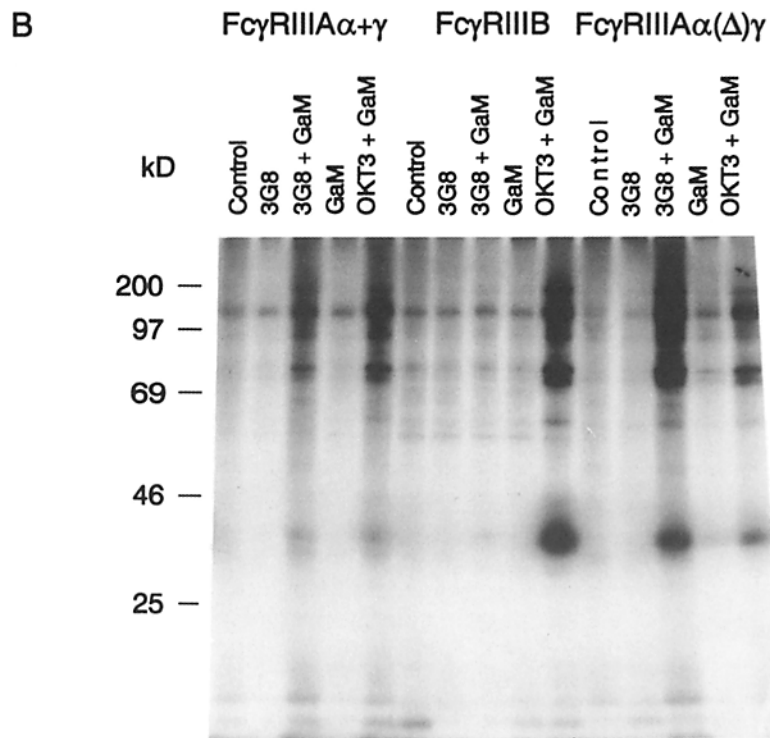
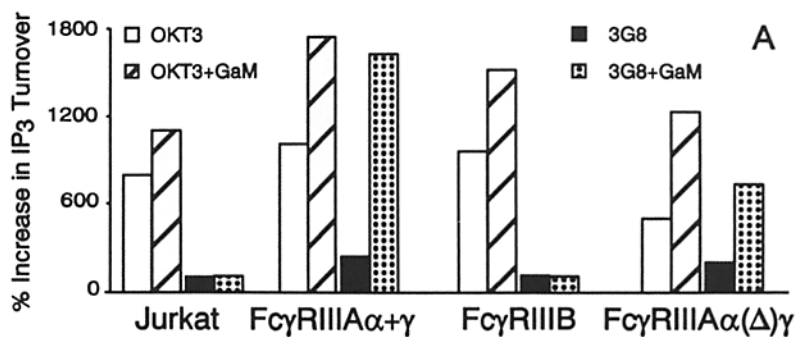
**Signal Transduction by Fc $\gamma$ RIII in P815 Cells.** Jurkat cells are a useful model to compare the stimulatory potency of the different isoforms of Fc $\gamma$ RIII, but since Fc $\gamma$ RIIIA is normally expressed in cells of the myeloid lineage (NK cells, macrophages, and mast cells), we have also analyzed its signal-transducing capabilities in the murine mastocytoma cell line P815. P815 cells express endogenous murine Fc $\gamma$ RII and Fc $\gamma$ RIII, but are deficient in the expression of Fc $\epsilon$ R1 (29, 30). To investigate in detail the functional properties of the different subunits of the receptor complex, P815 cells were transfected with Fc $\gamma$ RIIIA $\alpha$ + $\gamma$  and with a chimeric molecule composed of the ligand-binding (extracellular) domains of Fc $\gamma$ RIIIA $\alpha$  coupled to the transmembrane and cytoplasmic domain of Fc $\epsilon$ R1 $\gamma$  (Fc $\gamma$ RIIIA $\alpha$ / $\gamma$ ).

G418 resistant clones were selected and analyzed for the expression of the transfected cell-surface receptors by FACS<sup>®</sup> (Fig. 5). Receptor activation was performed using 3G8 and G $\alpha$ M F(ab')<sub>2</sub>, free of contaminating intact IgG. Functional analysis of the resulting P815 clones expressing Fc $\gamma$ RIIIA $\alpha$ + $\gamma$  and Fc $\gamma$ RIIIA $\alpha$ / $\gamma$ , respectively, reveal receptor-triggered transient increases in [Ca<sup>2+</sup>]<sub>i</sub>, and induction of PIP<sub>2</sub> hydrolysis (Fig. 6 B). These experiments validate the Jurkat cell model and confirm the ability of Fc $\gamma$ RIIIA $\alpha$ + $\gamma$  to elicit proximal signal transduction events in a myeloid background. As was found in Jurkat cells, the cytoplasmic domain of the  $\alpha$  chain is not necessary for signal transduction, requiring only the presence of the transmembrane and cytoplasmic domains of the  $\gamma$  chain to induce both transient changes in [Ca<sup>2+</sup>]<sub>i</sub> and induction of PIP<sub>2</sub> hydrolysis. The weaker response elicited by the chimeric Fc $\gamma$ RIIIA $\alpha$ / $\gamma$  upon activation with 3G8 can be explained by the decreased level of surface expression of this construct compared with Fc $\gamma$ RIIIA $\alpha$ + $\gamma$  (Fig. 5).

Finally, to investigate if the signal transduction events triggered by the Fc $\gamma$ RIIIA complex require cell-type specific molecules in addition to the  $\alpha$  and  $\gamma$  chains, we studied these hetero-oligomeric complexes in CHO cells. CHO cells expressing Fc $\gamma$ RIIIA $\alpha$  together with the murine Fc $\epsilon$ R1 $\gamma$  chain are incapable of triggering [Ca<sup>2+</sup>]<sub>i</sub> or PIP<sub>2</sub> hydrolysis after stimulation with antireceptor antibodies, or in combination with crosslinking G $\alpha$ R IgG F(ab')<sub>2</sub> (data not shown). These results indicate that activation through Fc $\gamma$ RIIIA complex is restricted to cell types of lymphoid and myeloid origin.

## Discussion

Progress in understanding the role of specific Fc $\gamma$ R<sub>s</sub> and their associated subunits in signal transduction has been hindered by the fact that most cell types express multiple types of Fc $\gamma$ R<sub>s</sub>. Attempts to trigger individual Fc $\gamma$ R<sub>s</sub> on a single cell type have relied upon the use of specific mAbs that recognize discrete classes of receptors. However, each Fc $\gamma$ R class represents multiple isoforms that differ in their cytoplasmic

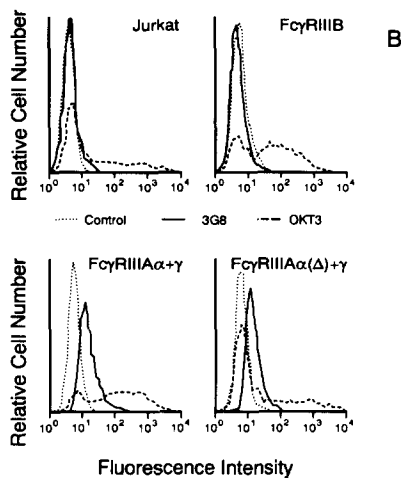
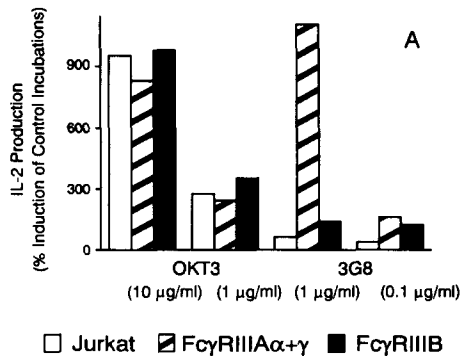


**Figure 3.** (A) Stimulation of PIP<sub>2</sub> hydrolysis in Jurkat cells expressing FcγRIII. Cells loaded with myo-[<sup>3</sup>H]-inositol were stimulated at 37°C with either OKT3 (1 μg/ml) alone (open bars), OKT3 and crosslinking (GαM IgG F(ab')<sub>2</sub>; 10 μg/ml, hatched bars), 3G8 (1 μg/ml) alone (solid bars), or 3G8 and crosslinking (dotted bars). The incubation time for mAbs and crosslinking Abs was 3 min each. Soluble inositol phosphates were assayed as described in Materials and Methods. Values represent percent increase in IP<sub>3</sub> production of nonstimulated cells. Mean values of separate experiments are shown (n = 3). SD never >10%. (B) Stimulation of FcγRIIIA in transfected Jurkat cells results in activation of a tyrosine kinase. [<sup>32</sup>P]orthophosphate-labeled cells were either unstimulated (control), stimulated with 3G8 (1.7 μg/ml), stimulated with 3G8 and crosslinked with GαM IgG F(ab')<sub>2</sub> (3.2 μg/ml; 3G8+GαM), stimulated with OKT3 (1.2 μg/ml) and crosslinking (OKT3+GαM) at 37°C. The incubation time for mAbs and crosslinking Abs was 2.5 min each. Lysates were immunoprecipitated with the antiphosphotyrosine antibody 4G10 and protein A sepharose CL4B, and subsequently analyzed by SDS-PAGE as described in Materials and Methods. Each lane represents ~5 × 10<sup>6</sup> cells.

domains (2, 3), resulting in the simultaneous crosslinking of multiple FcγRs. In addition, the mAbs themselves can trigger FcγRs through their Fc portions, confounding interpretation of results. These obstacles have made it very difficult to designate the role of one particular type of FcγR with a specific signal transduction event, and has resulted in conflicting results. This is particularly the case in neutrophils, where all three different types of FcγRs are expressed (2, 3), and where the spectrum of the proposed function in terms of signal transduction by FcγRIIIB is very broad. Some reports describe it as a completely nonfunctional receptor that mediates only ligand binding without any further implication for cell activation (31, 32). Other studies have concluded that FcγRIIIB is a receptor that is able to mediate signals only because of coexpression of additional classes of FcγRs (33), and some reports describe it as a fully functional cell-surface receptor signalling independently (34).

Several experimental approaches have attempted to dissect

the contribution of the different FcγRs on neutrophils to the overall signal transduction responses. F(ab) and F(ab')<sub>2</sub> fragments have been used to target specific FcγRs and to prevent possible receptor crosslinking due to Fc binding. Although this approach seems to be suitable, it requires those fragments to be essentially free of remaining intact IgG, since contaminating amounts of as little as 1% are still able to activate cells by receptor crosslinking with other FcγRs (35). The use of PI-PLC to deplete FcγRIIIB on neutrophils also fails since there is a remaining PI-PLC-resistant population of FcγRIIIB that still might interfere with other receptors by crosslinking (34). Experiments have also been done using neutrophils from patients with acquired paroxysmal nocturnal hemoglobinuria (PNH) which have a deficiency in the GPI membrane-attachment mechanism (7, 8). However, even those cells retain about 10% of the normal level of cell surface-expressed FcγRIIIB, which could contribute to FcγRII-FcγRIII crosslinking. Studies that have accounted for these



**Figure 4.** (A) Induction of IL-2 production. Nontransfected Jurkat cells (open bars), Jurkat cells expressing FcγRIIIAα+γ (hatched bars), or FcγRIIIB (solid bars) were stimulated with the indicated mAb in GαM IgG-coated microtiter wells in the presence of PMA (10 ng/ml) for 24 h as described in Materials and Methods. IL-2 secretion was determined by the ability of culture supernatants of stimulated cells to support the growth of IL-2-dependent CTLL cells. Values represent percent increase over nonstimulated control cells, and are mean values from triplicate determinations of one experiment representative of two others. (B) Stimulation of FcγRIIIA induces expression of IL-2Rα in transfected Jurkat cells. Flow cytometry of cells stimulated for 16 h with a control mAb (32.2, dotted lines), OKT3 (dashed lines), or 3G8 (solid lines). Cells were incubated with biotin-conjugated anti-IL-2Rα mAb and labeled with fluorescein-coupled streptavidin. Stimulation was performed using fixed crosslinking antibodies as described in Materials and Methods. PMA was present at nonstimulatory amounts of 10 ng/ml.

difficulties have concluded that FcγRIIIB on neutrophils is unable to mediate ADCC, phagocytosis,  $[Ca^{2+}]_i$  or  $PIP_2$  hydrolysis (31, 32, 36).

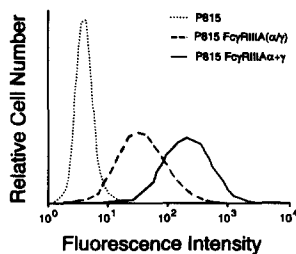
To clarify the signal transduction potential of FcγRIII in a simplified background, we transfected the cDNA of these receptors into different cell lines. Expression of FcγRIIIB was independent of coexpression of FcεRγ, and the receptor molecule was not found to be associated with any other cellular proteins approximating the *in vivo* situation for neutrophils. Our results strongly suggest that there is no evidence that FcγRIIIB is able to elicit signal transduction after ligand binding and subsequent crosslinking. Transmembrane signalling and stimulation of cell proliferation have been demonstrated for other GPI-linked proteins, most notably on T cells. Jurkat cells have been shown to be activated through transfected GPI-linked Thy-1 in the presence of TCR/CD3 (37). Under similar conditions, we are unable to elicit FcγRIIIB-dependent Jurkat cell activation. A recent report indicated that a number of different GPI-linked cell surface molecules are physically associated with the tyrosine kinase lck (38).

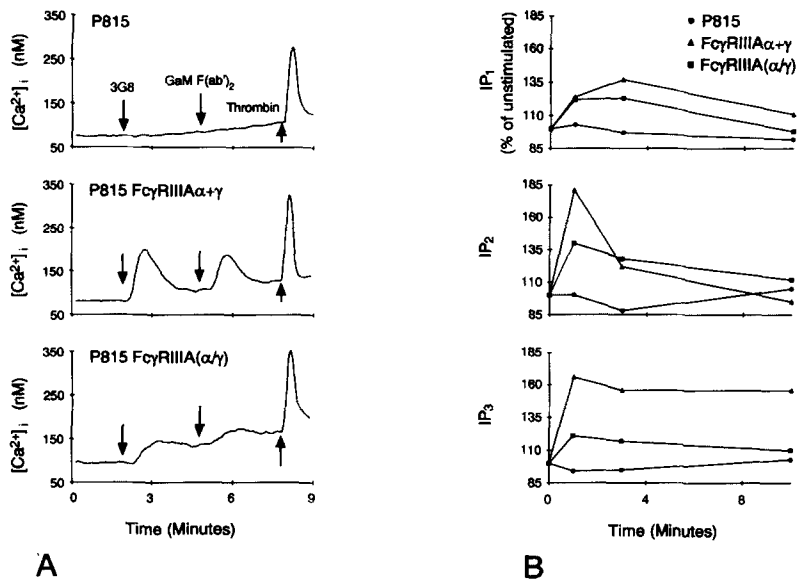
We have detected expression of lck in our transfected Jurkat cells, and did not see any induction of tyrosine phosphorylation after engagement of the GPI-linked FcγRIIIB.

In contrast, signal transduction by the FcγRIIIA complex has been studied mostly in NK cells (31, 32), where it is the only one type of FcγR expressed (39). A number of cell activation events have been reported to occur upon engagement of the FcγRIIIA complex, including proximal responses such as increases in  $[Ca^{2+}]_i$ ,  $PIP_2$  hydrolysis (31), as well as distal responses such as the transcriptional activation of specific cytokines like interferon-γ and TNF (32). It has been demonstrated that in NK cells, FcγRIIIAα is associated with either γ<sub>2</sub>, ζ<sub>2</sub>, or γζ (12, 13). This association is necessary to prevent the receptor complex from degradation during processing in the ER (16). Since both γ and ζ share extensive sequence homology, and the ζ chain has been shown to elicit signalling through the TCR/CD3 (40), FcεRIγ is likely to be the potential signal-transducing molecule of the FcγRIIIA complex. A T cell line that has the signal transduction machinery used by ζ might therefore represent a useful model to investigate functional properties of γ and its contributions to signal transduction through the FcγRIIIA complex.

In this report we present a functional model for FcγRIIIAα+γ in the T cell line Jurkat, a system that allows investigation of both proximal and distal signal transduction responses. The transfected FcγRIIIA complex was functional, and the signals obtained were qualitatively and quantitatively similar to those obtained by activation through the TCR/CD3 complex. This result further illustrates that TCR/CD3ζ and FcεRIγ share extensive sequence homology and have similar roles in receptor assembly, as well as in signal transduction. Several recent studies have demonstrated ζ chain cytoplasmic sequences to be necessary and sufficient to trigger T cell acti-

**Figure 5.** Cell surface expression of FcγRIIIA in transfected P815 cells. Nontransfected P815 cells (dotted line), P815 expressing FcγRIIIAα+γ (solid line), and P815 expressing FcγRIIIAα/γ (dashed line) were incubated with 3G8 followed by labeling with fluorescein-conjugated GαM F(ab')<sub>2</sub>. Nonspecific staining was assessed by using an isotype-matched negative control mAb 32.2, and was comparable with the P815 control (data not shown).





**Figure 6.** (A) Calcium mobilization of Fc $\gamma$ RIIIA in transfected P815 cells. Nontransfected P815, cells transfected with Fc $\gamma$ RIIIA $\alpha$ + $\gamma$ , or Fc $\gamma$ RIIIA( $\alpha$ / $\gamma$ ) loaded with fura-2/AM were stimulated with anti-Fc $\gamma$ RIII mAb (3G8, 0.1  $\mu$ g/ml), and subsequently crosslinked with G $\alpha$ M F(ab')<sub>2</sub> (10  $\mu$ g/ml). Murine thrombin (0.1 U/ml) was used as a positive control. Cells were analyzed for changes in [Ca<sup>2+</sup>]<sub>i</sub> by fluorometry as described in Materials and Methods. Stimuli were added at times indicated (arrows). (B) Stimulation of PIP<sub>2</sub> hydrolysis in P815 cells expressing Fc $\gamma$ RIIIA. Cells loaded with myo-[<sup>3</sup>H]-inositol were stimulated for 3 min at 37°C with 3G8 (1  $\mu$ g/ml). Soluble inositol phosphates were assayed as described in Materials and Methods. Values represent percent increase in IP production of nonstimulated cells and are mean values from triplicate determinations of one experiment representative of three others. SD never >10%.

vation when coupled to a heterologous extracellular domain. Thus, CD8/ $\zeta$ , CD4/ $\zeta$ , and IL-2R $\alpha$ / $\zeta$  chimeras are able to trigger proximal and distal signals upon crosslinking (20, 41, 44). Further experiments will be required to determine the possible differences in cell activation by  $\gamma$  as compared with  $\zeta$ .

By successfully expressing a functional Fc $\gamma$ R complex in a heterologous T cell system, we demonstrate the capability of this receptor to elicit signal transduction independent of NK cell-specific proteins. Stimulation of this receptor complex results in activation of both PIP<sub>2</sub> hydrolysis and tyrosine kinase activity. In addition, distal activation events such as the increase in IL-2R $\alpha$  expression and production of IL-2 confirm the physiologic manner of the receptor-induced signals. Our findings, that the signals transduced by the cytoplasmic deletion mutant Fc $\gamma$ RIIIA $\alpha$  ( $\Delta$ )+ $\gamma$  are indistinguishable from those generated by the wild-type Fc $\gamma$ RIIIA $\alpha$ + $\gamma$ , suggest that the  $\alpha$  cytoplasmic domain is not required for Fc $\gamma$ RIIIA-mediated signalling. In addition, the chimeric  $\alpha$ / $\gamma$  molecule demonstrates that the  $\gamma$  subunit couples ligand binding to intracellular signalling. Since a number of different tyrosine kinases such as fyn and lck have been found to be expressed in Jurkat cells (unpublished results), and can also be found to be activated by the CD8/ $\zeta$  (20),  $\gamma$  may also provide a direct link between Fc $\gamma$ RIIIA $\alpha$  and these tyrosine kinases.

Fc $\gamma$ RIIIA $\alpha$ + $\gamma$  is expressed on the cell surface of NK cells, macrophages, and mast cells. We have extended our studies using the murine myeloid mastocytoma cell line P815. Our results demonstrate that Fc $\gamma$ RIIIA $\alpha$ + $\gamma$  is functional in both lymphoid and myeloid backgrounds. We have recently identified an additional chain in the Fc $\gamma$ RIIIA $\alpha$ + $\gamma$  complex in murine mast cells, the  $\beta$  chain of the Fc $\epsilon$ R1 complex (43). This finding suggested the possibility that  $\beta$ -like molecules may be responsible for signalling for Fc $\gamma$ RIIIA receptors. However, coexpression of Fc $\gamma$ RIIIA $\alpha$ ,  $\beta$ ,  $\gamma$  in CHO cells did not reconstitute receptor signalling. These data further indicate that the signal transducing capability of Fc $\gamma$ RIIIA requires cell type-restricted molecules, in addition to  $\alpha$ ,  $\beta$ , and  $\gamma$ .

Our studies to date have dissected several functional domains for the Fc $\gamma$ RIIIA complex: (a) the extracellular domain of Fc $\gamma$ RIIIA $\alpha$  subunit provides ligand recognition (9-11); (b) the transmembrane domain of this subunit determines the half-life of the subunit in the ER and mediates  $\gamma$  chain assembly; (c) the transmembrane domain of the  $\gamma$  subunit prevents ER degradation of the  $\alpha$  subunit (16); and (d) the cytoplasmic domain of  $\gamma$  initiates intracellular signalling events. The mechanism by which the  $\gamma$  cytoplasmic domain initiates these events upon receptor crosslinking is now open for dissection.

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