Signal Transduction by FcγRIII (CD16) Is Mediated through the γ Chain

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Summary

To determine the functional role of the two isoforms of FcγRIII (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.

FcγRIIIA, a hetero-oligomeric receptor composed of a ligand-binding subunit α and dimeric γ 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, FcγRIIIB, which is normally attached to the cell surface via a glycosyl-phosphatidylinositol anchor, was incapable of transducing signals. After crosslinking, FcγRIIIA signalling was dependent only upon the γ chain. FcγRIIIA chimeras in which the α subunit transmembrane and cytoplasmic domains were substituted with the corresponding γ chain sequences functioned as well as wild-type hetero-oligomeric receptors. These data indicate that the ability of the FcγRIIIA complex to activate the appropriate pathways for cell activation is cell-type restricted and independent of the transmembrane and cytoplasmic domains of the α subunit. The presence of the γ chain is responsible for the assembly of, as well as the signal transduction by, the functional cell surface complex.

Binding of antibodies to effector cells through receptors recognizing their constant regions (FcRs) 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)1 (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 (FcγR) have been defined based upon their different protein and genomic structures: the high-affinity FcγRI (CD64), and the low-affinity FcγRs FcγRII (CD32) and FcγRIII (CD16) (3). Evidence suggests that FcγRs couple ligand binding to intracellular signalling events, but the contribution of each particular type of FcγR to the overall cell activation process is still unknown.

Although many early biochemical events such as stimulation of phospholipase C, mobilization of intracellular calcium ([Ca2+]i), 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 FcγRIII, 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 FcγRIIIA and FcγRIIIB, 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 FcγRIIIB is uniquely expressed on neutrophils (7, 8), while the other isoform, FcγRIIIA, is expressed on NK cells and on macrophages, and exists as a hetero-oligomeric receptor complex consisting of a ligand-binding α chain associated with disulfide-linked γ or ε chains (9–13). The associated γ or ε chains are required for cell surface expression of FcγRIIIA α, and are presumed to be involved in signal transduction (9–12). The γ and ε subunits

1 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 (FceRI), and the TCR/CD3 complex, respectively (14, 15).

To explore the capability of the different isoforms of FcγRIII to generate intracellular signaling 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 FcγRII.

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 FcγRIIIA and 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). [32P]orthophosphate (10 mCi/ml), and [3H]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-3ot,6c~-diphenylglycouril (Iodogen) was purchased from Pierce Chemical Co. (Rockford, IL). Phosphatidylinositol-specific phospholipase C (PI-PLC, from Boehringer Mannheim Corp.) was from Boehringer Mannheim Corp. (Indianapolis, IN). All other reagents were from Fluka Chem. Corp. (Ronnkonkoma, NY).

mAbs. The following antibodies were used: mouse anti-human CD3ε: OKT3 (IgG2a, Ortho Diagnostics Systems, Inc., Raritan, NJ); mouse anti-human CD16: 3G8 (IgG1, Dynal Inc., Great Neck, NY); mouse anti-human CD25 (IgG1, Medarex Inc., West Lebanon, NH); mouse anti-human IL-2 receptor α chain (IL-2Rα): CD25, IgG1, biotin conjugate; Boehringer Mannheim; mouse anti-phosphotyrosine: 4G10 (IgG2b/k; Upstate Biotechnology Inc., Lake Placid, NY); GmM IgG F(ab')2, 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% CO2. 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). cDNA encoding human FcγRIIIα and murine FcγRIγ were cloned together with the neomycin resistance gene into the expression vector pEXVT-3 (pEXV-3N/FcγRIIIα + γ) (19). A cytoplasmic deletion clone of FcγRIII was described previously (FcγRIIIB with Sε changed to Phe) (11). This mutated cDNA was cloned together with the cDNA encoding the γ chain and the neomycin resistance gene into pEXV-3 (pEXV-3N/FcγRIIIα(Δ) + γ). The chimeric FcγRIIIα/γ, which contains the extracellular domain of FcγRIIIα attached directly to the transmembrane and cytoplasmic portions of FcγRIγ, was constructed using PCR methods, and cloned together with the neomycin resistance gene into pEXV-3 (pEXV-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+ cells were isolated using the anti-FcγRIII mAb 3G8, and Dynabeads coated with GoM IgG (Dynal Inc., Great Neck, NY). All transfected Jurkat clones were kept in selective medium, and have been phenotype specific 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 (10/50 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 GoM IgG F(ab')2 (1 µg/ml; Tago Inc.). Cells were resuspended in 300 µl PBS/BSA and analyzed using a FACSScan® (Becton Dickinson & Co., San Jose, CA).

Cell-Surface Labeling and Immunoprecipitation. 107 cells were surface-labeled with 125I by using 1,3,4,6-tetrahydroxy-3ot,6c~-diphenyglycuril. 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 RoM IgG antibody (Pharmacl LKB Biotechnology, Inc., Piscatway, 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 ~7°C for 3 d.

Measurement of [Ca2+]i. Measurements of intracellular free calcium levels were performed with fura-2/AM. Cells (5 × 105) 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 CaCl2, 0.5 mM MgCl2, 5 mM KCl, 1 mM Na2HPO4, and 5 mM glucose. After 15 min of incubation at 37°C, the cell suspension was diluted to 106 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°C for 10 min. Fluorescence of the
stirred cellular suspension was continuously monitored with an Al-
phascan fluorimeter (Photon Technology International Inc., New
Brunswick, NJ). [Ca²⁺] was measured and computed as described
previously (21).

**Phosphoinositide Hydrolysis.** Jurkat cells or P815 cells were la-
beled with myo-[³H]-inositol (10 μCi/ml, $10^6$ cells/ml) for 6 h in
inositol-free RPMI 1640 supplemented with 10% dialyzed FCS.
The cells ($5 \times 10^6$/ml) were pre-equilibrated at 37°C and sequen-
tially stimulated with 3G8 (1 μg/ml) and GmM IgG F(ab')$_2$ (10
μg/ml) in the presence of 10 mM LiCl. The soluble inositol phos-
phates were extracted with TCA (200 μl) 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$_2$O and 10 ml 60 mM ammonium formate/5 mM disodium-
tetraborate, and elution was performed with increasing concen-
trations of ammonium formate (0.1–0.7 M) (22). The eluted radioac-
tivity was quantified by liquid scintillation counting in Aquasol
(NEN Du Pont, Boston, MA).

**Tyrosine Phosphorylation.** For tyrosine phosphorylation analysis,
$2 \times 10^6$ cells were incubated for 3 h at 37°C in 800 μl phosphate-
free DME supplemented with 0.5% dialyzed BSA and glutamine.
The cells were labeled with [³²P]orthophosphate (1 mCi/ml) for 4
h at 37°C, and stimulated with mAbs for 2.5 min. Cells were pelleted and resuspended in 300 μl lysis buffer (50
mM Hepes (pH 7.45), 150 mM NaCl, 1% digitonin, 5 mM EGTA,
50 μg/ml leupeptin, 10 μg/ml aprotinin, 2 μg/ml pepstatin, 1 mM
PMSF, 200 μM sodium orthovanadate, 20 mM NaF, and 20 mM
sodium pyrophosphate), incubated at 4°C for 1 h, and clarified
by centrifugation at 14,000 rpm for 15 min at 4°C. Immunoprecipi-
tations were carried out from supernatants by addition of 1 μg
antiphosphotyrosine mAb 4G10 for 16 h at 4°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°C for 16 h. Each lane represents $n=2 \times 10^6$ cells.
**IL2 Production and IL2R Expression.** Jurkat cells were incubated
with the indicated mAbs for 30 min at 4°C. After removal of
unbound antibody, the cell concentration was adjusted to $10^6$
cells/ml, and 200 μl aliquots were transferred to 96-well microtiter
plates that were precoated with Gc~M IgG and blocked with RPMI
plus 10% FCS. PMA and A23187 were added at final concentra-
tions of 10 ng/ml and 1 μM, respectively. After 36 h of culture,
IL2 activity was assessed by measuring [³H]thymidine uptake of
the IL2-dependent cell line CTLL in a 1:4 dilution of the test su-
pernatants. 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.

**Expression of Different Isomers of FcγRIII on Transfected Jurkat
Cell Lines.** The stable expression plasmids for FcγRIIIB,
FcγRIIIAα+γ and FcγRIIIAα(Δ)+γ 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 FcγRIII
were equally expressed on the transfected Jurkat cells (Fig.
1 A). Cell surface expression of FcγRIIIAα+ and FcγRIIIAα(Δ)
were obtained only by cotransfection with the γ chain. As
expected, FcγRIIIB was susceptible to digestion with PI-PLC,
while FcγRIIIAα+γ and FcγRIIIAα(Δ)+γ 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 complexs.
The FcγRIII isoforms were immunoprecipitated from the
lyses using a variety of antibodies (Fig. 1 B). 3G8, a mAb
directed against the extracellular domain of FcγRIII,
immunoprecipitated a broad band of protein from cells expressing
FcγRIIIB characteristic of this glycoprotein (Fig.
1 B, 3G8). Polyclonal antiserum raised against the cytoplasmic
domain of FcγRIIIAα did not react with extracts from the
same cells (data not shown).

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

The molecular appearance of the FcγRIII isoforms in Jurkat
cells reflects the situation in vivo, where on neutrophils
FcγRIIIB is expressed as a GPI-linked monomer (4–8), while
on NK cells or macrophages FcγRIIIA appears as a receptor
complex consisting of one membrane spanning α chain
associated with either a γ or δ heterodimer, or a γ/δ het-
erodimer (9, 12, 13).

**Crosslinking of FcγRIIA Leads to an Increase in [Ca²⁺]++.**
The FcγRIII-expressing Jurkat cells allowed us to determine
the signalling capacity of these molecules in the absence of
other FeRs. We therefore examined the ability of 3G8 to elicit
an increase in [Ca²⁺] in Jurkat cells transfected with FcγRIII
+ γ FcγRIIIAα(Δ)+γ or FcγRIIIB. Fig. 2 depicts
typical flurometry tracings obtained with the different Jurkat
clones upon stimulation of FcγRIII or TCR/CDS, and sub-
sequent crosslinking of the antibody-coated cell surface mol-
cules with GmM IgG F(ab')$_2$. Stimulation of the wild-type
FcγRIIIAα+γ complex and the cytoplasmic deletion clone

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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 GoM F(ab')2 (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 FcγRIIIA (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 FcγRIIB did not show any associated bands. The respective clones and antibodies used are indicated. Each lane represents ~10^7 cells. Positions of the molecular weight standards are indicated (kD).

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

Stimulation of FcγRIII Results in Activation of PIP_2 Hydrolysis and Tyrosine Kinase Pathways. Since the increases in [Ca^{2+}] that occur with TCR/CD3 stimulation are attributed to increases in inositol phosphates, we tested the ability of FcγRIIIB to induce PIP_2 hydrolysis by assessing changes in total soluble inositol phosphates after stimulation with 3G8 (Fig. 3 A). Stimulation of the FcγRIII 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_2 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. 3 A). These results are consistent with the calcium fluorometry data and confirm the ability of FcγRIIIA to activate PIP_2 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_2 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 antiphosphotyrosine antibodies from 32p-labeled cell extracts revealed that a small number of tyrosine phosphoproteins were present in all unstimulated cells. (Fig. 3 B, Control lane). Stimulation of cells with 3G8 or the crosslinking GoM IgG F(ab')2 alone had no effect in the different FcγRIII-expressing Jurkat cells. Subsequent crosslinking of 3G8 coated cells with GoM IgG F(ab')2 resulted in the appearance of a pattern of tyrosine phosphoproteins in cells expressing FcγRIIIAα+γ and FcγRIIIAα(Δ)+γ that was indistinguish-
receptor-mediated signals from the cell surface to the nucleus detected in cells expressing FcγRIII and in nontransfected linking. Thus FcγRIIIAα/β and FcγRIIIAβ/γ are both able from that seen after stimulation with OKT3 and crosslinking. Thus FcγRIIIα/β and FcγRIIIα(Δ)+γ 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 FcγRIII and in nontransfected control cells.

Stimulation of FcγRIII 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 Goat IgG (Fig. 4 A). Stimulation with 3G8 resulted in a comparable increase in IL-2 production, but only in the cells expressing either FcγRIIIα/β+γ (Fig. 4 A) or FcγRIIIα(Δ)+γ (data not shown).

Another gene that is used as an activation marker in T cells encodes the IL-2 receptor α chain (IL-2Ra, 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 transduction events, late activation events occur upon stimulation of FcγRIIIA, but not FcγRIIIIB, thus demonstrating its ability to couple to the relevant signal transduction pathways in a physiologic manner. The cytoplasmic domain of FcγRIIIAα subunit is not necessary for either the early or the late activation events examined here.

Signal Transduction by FcγRIII in P815 Cells. Jurkat cells are a useful model to compare the stimulatory potency of the different isoforms of FcγRIII, but since FcγRIIIA 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 FcγRII and FcγRIII, but are deficient in the expression of FcεRII (29, 30). To investigate in detail the functional properties of the different subunits of the receptor complex, P815 cells were transfected with FcγRIIIAα+γ and with a chimeric molecule composed of the ligand-binding (extracellular) domains of FcγRIIIAα coupled to the transmembrane and cytoplasmic domain of FcεRI (FcγRIIIAα/γ).

G418 resistant clones were selected and analyzed for the expression of the transfected cell-surface receptors by FACS® (Fig. 5). Receptor activation was performed using 3G8 and Goat F(ab')2, free of contaminating intact IgG. Functional analysis of the resulting P815 clones expressing FcγRIIIAα+γ and FcγRIIIAα/γ, respectively, revealed receptor-triggered transient increases in [Ca²⁺], and induction of PIP2 hydrolysis (Fig. 6 B). These experiments validate the Jurkat cell model and confirm the ability of FcγRIIIAα+γ to elicit proximal signal transduction events in a myeloid background. As was found in Jurkat cells, the cytoplasmic domain of the α chain is not necessary for signal transduction, requiring only the presence of the transmembrane and cytoplasmic domains of the γ chain to induce both transient changes in [Ca²⁺], and induction of PIP2 hydrolysis. The weaker response elicited by the chimeric FcγRIIIAα/γ upon activation with 3G8 can be explained by the decreased level of surface expression of this construct compared with FcγRIIIAα+γ (Fig. 5).

Finally, to investigate if the signal transduction events triggered by the FcγRIIIA complex require cell-type specific molecules in addition to the α and γ chains, we studied these hetero-oligomeric complexes in CHO cells. CHO cells expressing FcγRIIIAα together with the murine FcεRIγ chain are incapable of triggering [Ca²⁺], or PIP2 hydrolysis after stimulation with antireceptor antibodies, or in combination with crosslinking Goat IgG F(ab')2 (data not shown). These results indicate that activation through FcγRIIIA complex is restricted to cell types of lymphoid and myeloid origin.

Discussion

Progress in understanding the role of specific FcγRs and their associated subunits in signal transduction has been hindered by the fact that most cell types express multiple types of FcγRs. Attempts to trigger individual FcγRs on a single cell type have relied upon the use of specific mAbs that recognize discrete classes of receptors. However, each FcγR class represents multiple isoforms that differ in their cytoplasmic...
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')2 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 IgGs, 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
Jurkat cells have been shown to be activated through transdifficulties have concluded that FcR IIIB on neutrophils is binding and subsequent crosslinking. Transmembrane signalling and subsequent crosslinking in the ER (16). Since both 3' and γ chain have been shown to elicit signalling similar to those obtained by activation through the TCR/CD3 (40). FcRγ in transfected Jurkat cells, and did not see any induction of tyrosine phosphorylation after engagement of the GPI-linked FcR IIIB.

To clarify the signal transduction potential of FcγR III in a simplified background, we transfected the cDNA of these receptors into different cell lines. Expression of FcγR IIIB was independent of coexpression of FceRγ, 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 FcγR IIIB 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 FcγR IIIB-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).

In contrast, signal transduction by the FcγR III A 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 FcγR III A complex, including proximal responses such as increases in [Ca2+]i, PIP₂ 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, FcγR III Aα is associated with either γ2, γ3, or γ5 (12, 13). This association is necessary to prevent the receptor complex from degradation during processing in the ER (16). Since both γ and s chain share extensive sequence homology, and the s chain has been shown to elicit signalling through the TCR/CD3 (40), FcRγ is likely to be the potential signal-transducing molecule of the FcγR III A complex. A T cell line that has the signal transduction machinery used by s might therefore represent a useful model to investigate functional properties of γ and its contributions to signal transduction through the FcγR III A complex.

In this report we present a functional model for FcγR III Aα+γ in the T cell line Jurkat, a system that allows investigation of both proximal and distal signal transduction responses. The transfected FcγR III A 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/CD3γ and FcRγ share extensive sequence homology and have similar roles in receptor assembly, as well as in signal transduction. Several recent studies have demonstrated s chain cytoplasmic sequences to be necessary and sufficient to trigger T cell acti-
vation when coupled to a heterologous extracellular domain. Thus, CD8/γ, CD4/γ, and IL-2Rα/γ 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 γ as compared with β.

By successfully expressing a functional FcγR 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 PIP₂ hydrolysis and tyrosine kinase activity. In addition, distal activation events such as the increase in IL-2Rα 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 FcγRIIIα (Δ) + γ are indistinguishable from those generated by the wild-type FcγRIIIα + γ, suggest that the α cytoplasmic domain is not required for FcγRIIIA-mediated signalling. In addition, the chimeric α/γ molecule demonstrates that the γ 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/γ (20), γ may also provide a direct link between FcγRIIIα and these tyrosine kinases.

FcγRIIIAα + γ 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 FcγRIIIAα + γ is functional in both lymphoid and myeloid backgrounds. We have recently identified an additional chain in the FcγRIIIAα + γ complex in murine mast cells, the β chain of the FceRI complex (43). This finding suggested the possibility that β-like molecules may be responsible for signalling for FcγRIIIA receptors. However, coexpression of FcγRIIIAα, β, γ in CHO cells did not reconstitute receptor signalling. These data further indicate that the signal transducing capability of FcγRIIIA requires cell type-restricted molecules, in addition to α, β, and γ.

Our studies to date have dissected several functional domains for the FcγRIIIA complex: (a) the extracellular domain of FcγRIIIα 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 γ chain assembly; (c) the transmembrane domain of the γ subunit prevents ER degradation of the α subunit (16); and (d) the cytoplasmic domain of γ initiates intracellular signalling events. The mechanism by which the γ cytoplasmic domain initiates these events upon receptor crosslinking is now open for dissection.

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