

Role for a Glycan Phosphoinositol Anchor in Fc γ Receptor Synergy

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Abstract. While many cell types express receptors for the Fc domain of IgG (Fc γ R), only primate polymorphonuclear neutrophils (PMN) express an Fc γ R linked to the membrane via a glycan phosphoinositol (GPI) anchor. Previous studies have demonstrated that this GPI-linked Fc γ R (Fc γ RIIIB) cooperates with the transmembrane Fc γ R (Fc γ RIIA) to mediate many of the functional effects of immune complex binding. To determine the role of the GPI anchor in Fc γ receptor synergy, we have developed a model system in Jurkat T cells, which lack endogenously expressed Fc γ receptors. Jurkat T cells were stably transfected with cDNA encoding Fc γ RIIA and/or Fc γ RIIIB. Cocrosslinking the two receptors produced a synergistic rise in intracytoplasmic calcium ([Ca²⁺]_i) to levels not reached by stimulation of either Fc γ RIIA or Fc γ RIIIB alone. Synergy was achieved by prolonged entry of extracellular Ca²⁺. Cocrosslinking Fc γ RIIA with CD59 or CD48,

two other GPI-linked proteins on Jurkat T cells also led to a synergistic [Ca²⁺]_i rise, as did crosslinking CD59 with Fc γ RIIA on PMN, suggesting that interactions between the extracellular domains of the two Fc γ receptors are not required for synergy. Replacement of the GPI anchor of Fc γ RIIIB with a transmembrane anchor abolished synergy. In addition, tyrosine to phenylalanine substitutions in the immunoreceptor tyrosine-based activation motif (ITAM) of the Fc γ RIIA cytoplasmic tail abolished synergy. While the ITAM of Fc γ RIIA was required for the increase in [Ca²⁺]_i, tyrosine phosphorylation of crosslinked Fc γ RIIA was diminished when cocrosslinked with Fc γ RIIIB. These data demonstrate that Fc γ RIIA association with GPI-linked proteins facilitates Fc γ R signal transduction and suggest that this may be a physiologically significant role for the unusual GPI-anchored Fc γ R of human PMN.

THE binding of immune complexes by polymorphonuclear neutrophils (PMN)¹ receptors for the Fc domain of IgG (Fc γ receptors) induces essential host defense and inflammatory responses such as adhesion, phagocytosis of antibody-coated microorganisms, degranulation, and the respiratory burst (33, 38). PMN activation by immune complexes is important in the pathology of serum sickness, the Arthus reaction, acute glomerulonephritis, rheumatoid arthritis, and other idiopathic inflammatory disorders as well as in host defense against infection. The Fc γ receptors are a family of hematopoietic cell receptors that share structurally related ligand-binding domains for the Fc portion of immunoglobulins, but which differ in their transmembrane and intracellular domains (for review see 16, 33). These varying cytoplasmic tails pre-

sumably give rise to distinct intracellular signals to provide diversity of function.

Primate PMN are unique, because in addition to the transmembrane Fc γ R, Fc γ RIIA, they express the only known eukaryotic nontransmembrane Fc γ R, the glycan phosphoinositol (GPI)-linked Fc γ RIIIB. Ligand binding by transmembrane Fc γ RIIA initiates a tyrosine kinase cascade dependent upon the cytoplasmic tail of this receptor, which contains one copy of an immunoreceptor tyrosine-based activation motif (ITAM) (11, 27), a substrate for phosphorylation by members of the src tyrosine kinase family. The phosphorylated ITAM of Fc γ RIIA can bind to and activate syk tyrosine kinase, which subsequently activates a number of effector pathways (16). In contrast, little is known about the signaling mechanisms of Fc γ RIIIB, the most abundant PMN Fc γ receptor. Some studies have suggested an inability of Fc γ RIIIB to transduce signals independently. These studies, taken together with this receptor's lack of a cytoplasmic domain, have led to the concept that Fc γ RIIIB is primarily an Fc-binding molecule that aids in immune complex presentation to Fc γ RIIA (1, 13). However, evidence now suggests that Fc γ RIIIB is able to mediate intracellular signaling events, such as the activation of the src family member hck and induction of intracellular

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1. *Abbreviations used in this paper:* [Ca²⁺]_i, intracytoplasmic Ca²⁺ concentration; GPI, glycan phosphoinositol; ITAM, immunoreceptor tyrosine-based activation motif; PLC, phospholipase C; PMN, polymorphonuclear neutrophils.

calcium fluxes (14, 19, 39, 49). Moreover, Fc γ RIIB cooperates with Fc γ RIIA in PMN activation. When ligated together, as would occur when PMN bind immune complexes, Fc γ RIIA and Fc γ RIIB synergize to activate the respiratory burst and to increase intracytoplasmic calcium (44, 47).

Despite the importance of the cooperation between Fc γ RIIA and Fc γ RIIB for PMN function, its mechanism is not understood. As primary, terminally differentiated, nondividing cells, PMN are exceedingly resistant to genetic and cell biological manipulations which have aided characterization of receptor function in other systems. We developed a model system to dissect the functional roles and domains of Fc γ RIIA and Fc γ RIIB in Jurkat T cells, which lack endogenous Fc γ receptors but are fully competent for tyrosine kinase signaling. In transfected Jurkat T cells, the PMN Fc γ receptors synergized to induce a rise in intracytoplasmic Ca²⁺ concentration ([Ca²⁺]_i) that was greater and more prolonged than from ligation of either receptor individually. This was identical to the effect of coligation of these receptors in PMN (44). The synergistic calcium rise required the influx of extracellular calcium and depended upon the GPI anchor of Fc γ RIIB, since a mutant in which the GPI anchor was replaced by the transmembrane domain of CD7 was unable to synergize with Fc γ RIIA. Moreover, crosslinking other GPI-linked proteins on Jurkat T cells with Fc γ RIIA also led to a synergistic increase in [Ca²⁺]_i. The increase in [Ca²⁺]_i also required the tyrosines of the Fc γ RIIA ITAM. Surprisingly, we found that phosphorylation of the ITAM was diminished under conditions that led to the synergistic calcium flux and that the kinetics of PLC- γ 1 phosphorylation was not altered by the replacement of the GPI anchor of Fc γ RIIB with the transmembrane domain of CD7. Thus, synergy between Fc γ R requires the GPI anchor of Fc γ RIIB, but not for an increase in Fc γ RIIA-dependent tyrosine kinase signaling. We hypothesize instead that the role for the GPI anchor of Fc γ RIIB is to sequester Fc γ RIIA into specialized membrane domains where signal transduction by the ITAM is altered. This could provide a further level of modulation of activation signals from immune complex binding and may explain many of the functions of the unusual GPI-linked Fc γ R of primate PMN. Moreover, this could be a general mechanism by which GPI anchored proteins affect signal transduction from transmembrane receptors.

Materials and Methods

Cells and Antibodies

The human Jurkat T cells (American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing 10% heat-inactivated FCS (Hyclone, Logan, UT), 2 mM L-glutamine, 0.1 mM NEAA, 50 mM 2-mercaptoethanol, and 100 μ g/ml penicillin and streptomycin under a 5% CO₂ atmosphere. The bulk population was cloned before transfection to minimize heterogeneity of the population. Human PMN were freshly purified from the peripheral blood of healthy donors as described (5). The following mAbs were used in this study: IV.3 (anti-CD32, anti-Fc γ RII; 26), 3G8 (anti-CD16, anti-Fc γ RIII; 9), IH4 (anti-CD55, anti-DAF; 8), MEM-43 (anti-CD59, anti-Protectin), 10G10 (anti-CD59; kindly provided by Dr. Marilyn Telen, Duke University, Durham, NC), MEM-102 (anti-CD48; Harlan Bioproducts, Indianapolis, IN), IIIA5 (anti-Fc γ RII; kindly provided by Dr. Jurgen Frey, Universität Bielefeld), and mouse IgG_{2b} isotype control (Sigma Chemical Co., St. Louis, MO). To crosslink primary antibodies, goat F(ab')₂ fragments specific for mouse F(ab') or goat F(ab')₂ fragments

specific for mouse IgG₁ or mouse IgG_{2b} (Sigma Chemical Co) were used. Antibody fragments of IV.3, 3G8, or 10G10 were made by standard methods or purchased (Medarex, Annandale, NJ). For FACS[®] analysis, bound mAbs were detected using FITC-conjugated goat F(ab')₂ fragments specific for mouse F(ab') (Sigma Chemical Co.). Anti-phospholipase C γ -1 (PLC- γ 1) was purchased from Upstate Biotechnology (Lake Placid, NY) or Transduction Laboratories (Lexington, KY). Anti-phosphotyrosine (Upstate Biotechnology) was detected with HRP-conjugated goat antibodies specific for mouse IgG_{2b} (Caltag Laboratories, So. San Francisco, CA).

Fc γ RIIA and Fc γ RIIB Expression Constructs and Transfection into Jurkat T Cells

The oligos 5'-CCTGAATTCCTCCGGATATCTTTGGTGAC-3' and 5'-AGAGGATCCGCTGCCACTGCTCTTATTAC-3' were used to amplify the human Fc γ RIIB (CD16) cDNA by RT-PCR of human PMN mRNA (24). The resulting product was digested with EcoRI and HindIII and ligated into similarly digested vectors, pBluescript II SK+/-, pRcCMV, and pCEP4 (Invitrogen, San Diego CA). The intactness of the cDNA was verified by DNA sequencing (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit; Perkin Elmer, Foster City, CA). The Fc γ RIIB/CD7 construct was made by ligating a HindIII/MluI fragment of the CD16/CD7/syk construct (kindly provided by Dr. Brian Seed, Harvard Medical School, Boston, MA; (20)) and a MluI/NotI adaptor (annealed oligonucleotides 5'-CGCGTTAATAGATCGATGC-3' and 5'-GGCCGCATCGATCTATTAA-3' [stop codons underlined]) into HindIII/NotI-digested pRcCMV. This construct encodes the Fc γ RIIB extracellular domain joined with a CD7 transmembrane domain. The cDNA was verified by DNA sequencing. The cDNAs encoding Fc γ RIIA and Fc γ RIIA with both ITAM tyrosines in the cytoplasmic tail mutated to phenylalanine were prepared as described (7, 27) and cloned into pRcCMV and pCEP4.

The resulting plasmids were introduced into clones of Jurkat T cells by electroporation. Cells (10⁷) in 400 μ l HEBS (25 mM Hepes, pH 7.05, 140 mM NaCl, 750 mM Na₂HPO₄) and plasmid (30 μ g in 100 μ l HEBS) were added to a 0.4-mm-gap width cuvette and electroporated at 1,000 μ F, 330 v (Electroporator II; Invitrogen). After electroporation, cells were cultured for 36 to 48 h in normal propagation media. Cells were transferred to selective media (propagation media plus 1.4 mg/ml geneticin/G418 [Gibco Laboratories] and/or 600 μ g/ml hygromycin B [Boehringer Mannheim, Indianapolis, IN]) and cultured for 2 to 3 wk. High protein-expressing cell populations were selected by fluorescence-activated cell sorting using mAb IV.3 or mAb 3G8. Briefly, cells (10⁶) were resuspended in 50 μ l PBS/5% FCS with 1 μ g antibody and incubated on ice for 45 min. Cells were washed and then incubated an additional 30 min with F(ab')₂ fragments of goat anti-mouse IgG-FITC (Sigma Chemical Co.). Cells were analyzed on a flow cytometer (Coulter Electronics, Hialeah, FL) or sorted using a fluorescence-activated cell sorter (Becton Dickinson, Palo Alto, CA). All cDNAs were introduced into at least two different Jurkat clones and all experiments yielded equivalent results in all clones.

[Ca²⁺]_i Measurements

Jurkat transfectants were loaded with 3 μ M Fura 2-AM (Molecular Probes, Eugene, OR) in RPMI 1640/10% FCS for 40 min in the dark at 37°C. PMN were loaded with 5 μ M Fura-2 AM in Hanks Balanced Salt Solution (HBSS; Gibco Laboratories), 1 mM MgCl₂, 1 mM CaCl₂, and 1% vol/vol human serum albumin (HBSS++) for 25 min in the dark at 37°C. Cells (6 \times 10⁶) were washed once, resuspended in RPMI 1640/10% FCS or HBSS++ containing the appropriate mAbs, and incubated 30 min on ice. Cells were washed three times and resuspended in 2 ml calcium buffer (25 mM Hepes, pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1 mg/ml D-glucose, 1 mg/ml BSA, 1 mM CaCl₂, 0.5 mM MgCl₂). Changes in fluorescence, using excitation wavelengths of 340 and 380 nm and the emission wavelength of 510 nm, were measured with a spectrofluorimeter (F-2000; Hitachi Instruments, Danbury, CT) equipped with a thermostatic cuvette holder maintained at 37°C. Cells were warmed to 37°C for 5 min and added to the cuvette; then 10 μ l mouse F(ab') specific goat F(ab')₂ fragments were added. Intracellular calcium concentrations were calculated as described (36).

Receptor Crosslinking, Immunoprecipitation, and Western Blots

Cells (1–2 \times 10⁷) were incubated in RPMI 1640/10% FCS containing the mAb IV.3 (15 μ g/ml) or the mAbs IV.3 and 3G8 (15 μ g/ml each) for 30

min on ice. Cells were washed three times, resuspended in 0.5 ml RPMI 1690 with 10% FCS, and then warmed to 37°C for 10 min. Crosslinking mouse F(ab')₂ specific goat F(ab')₂ fragments (20 μl) were added for various times. Cells were lysed with an equal volume of 2× lysis buffer (100 mM Tris-HCl, pH 7.4, 2% NP-40, 0.5% deoxycholate, 300 mM NaCl, 2 mM EDTA, 2 mM NaF, 250 μM Na₃VO₄, 1 mM Na₂MoO₄, 1 mM Na₂H₂P₂O₇, 10 ng/ml calyculin, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 15 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride) at 4°C. Samples were centrifuged 5 min at 14,000 g. Resulting supernatants were rotated overnight with 75 μl of a 1:1 slurry of Gamma Bind plus Sepharose (Pharmacia Biotech, Piscataway, NJ). For PLC-γ1 immunoprecipitations, 10 μl of polyclonal antibodies were added to each sample. Beads were washed extensively and resuspended in reducing cocktail (50% vol/vol glycerol, 250 mM Tris-HCl, pH 6.8, 5% wt/vol SDS, 570 mM 2-mercaptoethanol, bromophenol blue). Samples were boiled for 5 min and then subjected to SDS-PAGE and electrotransfer onto Immobilon-P (Milipore, Bedford, MA) membranes. Blots were probed with anti-phosphotyrosine, anti-FcγRII (III A5), or anti-PLC-γ1. Bound antibodies were detected with HRP-conjugated mouse specific goat antibodies. Antibody reactive protein was visualized using enhanced chemiluminescence (ECL; Amersham Intl., Arlington Heights, IL). Tyrosine phosphorylation of FcγRIIA or PLC-γ1 under different conditions was compared by normalizing the amount of phosphorylation, determined by densitometry of the anti-phosphotyrosine blots, to the amount of protein precipitated, as determined by reprobing the same blots with antibodies to the relevant protein. Multiple experiments were combined for analysis by comparing all experimental conditions to the ratio obtained for wild-type receptors in the same experiment.

Results

Cocrosslinking FcγRIIA and FcγRIIB Results in a Synergistic [Ca²⁺]_i Rise

Jurkat T cells, which do not express endogenous Fcγ receptors, were stably transfected with the cDNAs encoding FcγRIIA and FcγRIIB (J2/3; Fig. 1, *top*). In addition, stable transfectants were made which express FcγRIIA along with a chimeric receptor consisting of the extracellular portion of FcγRIIB coupled to the transmembrane domain of CD7 (J2/3-CD7; Fig. 1, *middle*). A third transfectant was made that expresses FcγRIIB and an FcγRIIA receptor in which the tyrosines (Y²⁸² and Y²⁹⁸) of the ITAM have been mutated to phenylalanines (27; J2Y→F/3, Fig. 1, *bottom*). FACS[®] analysis indicated that each mutant receptor is expressed at a level at least comparable to that of the corresponding wild-type receptor (Fig. 1).

Previous studies in PMN have shown that FcγRIIA and FcγRIIB in PMN cooperate to generate a calcium flux that is greater than the sum of the calcium fluxes generated by crosslinking either receptor individually (44). In addition, it has been shown that Jurkat cells that were stably transfected with FcγRIIA are able to flux calcium after receptor ligation (15), suggesting the signaling machinery used by Fcγ receptors is functional in these cells. Therefore we compared [Ca²⁺]_i in J2/3 cells after crosslinking FcγRIIA and FcγRIIB individually or after crosslinking both receptors together, using a F(ab')₂ crosslinking antibody. Crosslinking FcγRIIA resulted in a significant, short lived rise in [Ca²⁺]_i (Fig. 2, *top*). In contrast, crosslinking FcγRIIB alone resulted in a slow rise in [Ca²⁺]_i with a magnitude lower than for FcγRIIA (Fig. 2, *top*). When both FcγR were crosslinked together, there was an increase in the maximum [Ca²⁺]_i rise and a prolongation of the increase (Fig. 2, *top*). Synergy did not require the Fc fragment of either anti-FcγRII or -FcγRIII mAb, since similar results were obtained by using the F(ab) fragment of the mAb IV.3 and the F(ab')₂ fragment of the mAb

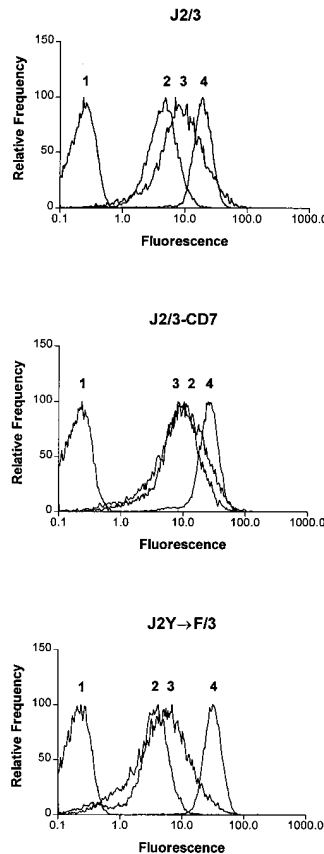


Figure 1. Fluorescent flow cytometric analysis of FcγR expression. Jurkat T cells (10⁶) expressing various Fcγ receptors were resuspended in 50 μl PBS/5% FCS with 1 μg of the mAb IV.3 (2), specific for FcγRIIA, mAb 3G8 (3), specific for FcγRIIB, or the mAb MEM-43 (4), specific for CD59. Cells were also stained with a negative control antibody (1). Cells were washed and then stained with F(ab')₂ fragments of FITC-conjugated goat anti-mouse antibodies and then analyzed by FACS[®]. Cells expressing wild-type FcγRIIA and FcγRIIB (J2/3; *top*), wild-type FcγRIIA and the chimeric FcγRIIB/CD7 (J2/3-CD7; *middle*), or wild-type FcγRIIB and the mutant FcγRIIA where the tyrosines within the ITAM (Y²⁸² and Y²⁹⁸) are changed to phenylalanine (J2Y→F/3; *bottom*) are shown.

3G8 (data not shown). Neither the addition of antibodies specific for Fcγ receptors alone nor the crosslinking goat F(ab')₂ fragments alone induced a rise in [Ca²⁺]_i (Fig. 2, *top* and data not shown). In PMN, crosslinking FcγRIIB is able to mediate a rise in intracellular calcium by itself. This difference between the Jurkat transfectants and PMN is most likely due to the level of FcγRIIB expression. In PMN, FcγRIIB is extremely abundant on the cell surface (12, 13). Phosphatidylinositol-specific phospholipase C (PLC) treatment of PMN, an enzyme that cleaves GPI-linked proteins and that removes 80% of the FcγRIIB from the cell surface, abolishes the rise in [Ca²⁺]_i after FcγRIIB crosslinking (35, and data not shown). Nonetheless, the expression level of FcγRIIB in the transfected Jurkat cells was sufficient to produce a synergistic rise in [Ca²⁺]_i.

To determine if the synergistic calcium response required bridging of FcγRIIA and FcγRIIB together or whether the augmentation in [Ca²⁺]_i could be achieved by simultaneously crosslinking each Fcγ receptor individually, isotype-specific secondary crosslinking antibodies were used (Fig. 2, *middle*). FcγRIIA was crosslinked with IV.3, an IgG_{2b} mAb, and goat F(ab')₂ fragments specific for mouse IgG_{2b} and FcγRIIB was crosslinked with 3G8, an IgG₁ mAb, and goat F(ab')₂ fragments specific for mouse IgG₁. When both Fcγ receptors were individually and simultaneously crosslinked, no synergistic rise in [Ca²⁺]_i was found (Fig. 2, *middle*), paralleling results found in PMN (44). In fact, the resulting rise in [Ca²⁺]_i appeared to be additive of the rises obtained by crosslinking both Fcγ receptors individually (Fig. 2, *middle*).

To show specificity of the synergy, cells were incubated

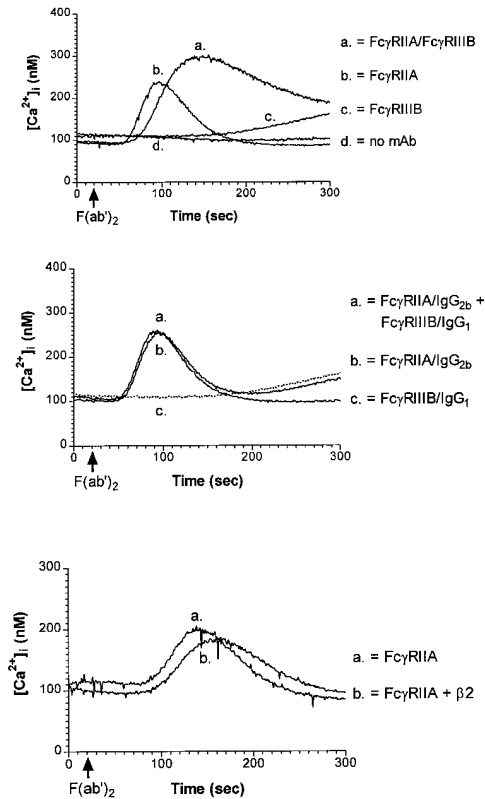


Figure 2. Changes in the $[Ca^{2+}]_i$ after crosslinking Fc γ R. Fura 2-AM pre-loaded J2/3 cells were incubated 30 min with the mAb IV.3 (anti-Fc γ RII, IgG $_{2b}$), the mAb 3G8 (anti-Fc γ RIIIB, IgG $_1$), or both these mAbs (*top* and *middle*). J2/3 cells also were incubated with mAb IV.3 and the mAb IB4, specific for β 2 integrins (*bottom*). F(ab') $_2$ fragments of goat anti-mouse antibodies (*top* and *bottom*), F(ab') $_2$ fragments of goat anti-mouse IgG $_1$ (*middle*), or F(ab') $_2$ fragments of goat anti-mouse IgG $_{2b}$ (*middle*) were added to crosslink Fc γ receptors at 20 s. Each curve is representative of at least three independent experiments. When Fc γ RIIA was crosslinked with mAb IV.3/anti-IgG $_1$ or Fc γ RIIIB was crosslinked with mAb 3G8/anti-IgG $_{2b}$, no rise in $[Ca^{2+}]_i$ resulted, demonstrating specificity of the secondary antibodies (data not shown). No rise in $[Ca^{2+}]_i$ resulted from the addition of secondary antibodies alone (data not shown).

with anti-Fc γ RII mAb IV.3 and the mAb IB4, specific for β 2 (CD18) integrins (Fig. 2, *bottom*). The β 2 integrin LFA-1 is expressed at a level similar to the transfected Fc γ RIIIB (data not shown). Moreover, LFA-1 synergizes with the ITAM-containing T cell antigen receptor to prolong an increase in $[Ca^{2+}]_i$ (45). However, there was no synergy between LFA-1 and Fc γ RIIA for $[Ca^{2+}]_i$ rise. This result indicates that signaling through Fc γ RIIA is augmented when cocrosslinked to Fc γ RIIIB, as would occur under physiological conditions where both Fc γ receptors are ligated by immune complexes.

The GPI Anchor Is Necessary and Sufficient for the Contribution of Fc γ RIIIB to Synergy

Primate PMN are the only cells that express a GPI-anchored Fc γ receptor (32). To determine whether the GPI anchor was necessary for Fc γ RIIIB contribution to the synergistic increase in $[Ca^{2+}]_i$, stable transfectants were made ex-

pressing Fc γ RIIA and a chimeric Fc γ RIIIB with the GPI anchor replaced by the transmembrane domain of CD7 (J2/3-CD7; Fig. 1, *middle*). When Fc γ RIIA and Fc γ RIIIB/CD7 were crosslinked together in these cells, the $[Ca^{2+}]_i$ rise was similar to the rise generated when Fc γ RIIA was crosslinked alone without any synergy from Fc γ RIIIB (Fig. 3, *middle*). The inability of the chimeric Fc γ RIIIB/CD7 molecule to contribute to the synergistic $[Ca^{2+}]_i$ rise was not due to inadequate expression of this protein, since the Fc γ RIIIB/CD7 molecule was expressed at a greater level than the wild-type Fc γ RIIIB (Fig. 1, *top* and *middle*). This experiment demonstrates that the GPI anchor is necessary for the synergistic $[Ca^{2+}]_i$ rise.

To determine whether any aspect of the extracellular Ig domains of Fc γ RIIIB rise were required for the synergistic $[Ca^{2+}]_i$ rise, other GPI-linked proteins expressed by Jurkat cells were cocrosslinked with Fc γ RIIA. CD48 (not shown) and CD59 (protectin) (Fig. 1) are both expressed by parental Jurkat cells and by each of the transfectants at levels equal to or greater than Fc γ RIIIB. When these GPI-linked proteins, CD59 (Fig. 3, *top*) and CD48 (not shown), were cocrosslinked with Fc γ RIIA, a synergistic rise in $[Ca^{2+}]_i$ also occurred in Jurkat cells transfected with Fc γ RIIA alone (data not shown), in J2/3 cells (Fig. 3, *top*), and in J2/3-CD7 cells (Fig. 3, *middle*). In all of these cells, ligation of CD59 alone produced a $[Ca^{2+}]_i$ rise similar to that elicited by crosslinking Fc γ RIIIB alone (Fig. 3, *top*, and data not shown).

These experiments demonstrate that the GPI anchor of Fc γ RIIIB is required for Fc γ R cooperation but that other extracellular domains will substitute for Fc γ RIIIB when cocrosslinked with Fc γ RIIA. This is strong evidence against the hypothesis that interaction between the extracellular domains of the receptors is required for synergy, as has been proposed for Fc γ RIIA and Fc γ RIIIB interaction with the β 2 integrin CR3 (for review see 30). Moreover, since these cells do not express CR3, this experiment shows that Fc γ R synergy can occur without this PMN integrin.

Synergy in PMN between Fc γ RIIA and Fc γ RIIIB was found for the rise in $[Ca^{2+}]_i$ (data not shown and 44), the respiratory burst (data not shown and 44, 47, 49), and degranulation (data not shown). To determine if the synergistic rise in $[Ca^{2+}]_i$ could also be obtained in PMN with other GPI-anchored proteins, Fc γ RIIA and CD59 were cocrosslinked and a prolongation in the rise $[Ca^{2+}]_i$ was found (Fig. 3, *bottom*). The synergistic rise in $[Ca^{2+}]_i$ with Fc γ RIIA and CD59 was not as pronounced as with Fc γ RIIIB and Fc γ RIIA. No significant synergy between Fc γ RIIA and CD59 was found in assays of degranulation or respiratory burst. This was true for CD48, CD55, and CD66b, other GPI-linked proteins on PMN, as well (data not shown). This is most likely due to a lower level of expression of these GPI-anchored proteins on PMN as compared to Fc γ RIIIB (CD59 has ~13% of the expression of Fc γ RIIIB, CD48 has 1%, CD55 has 6%, and CD66b has 9%; data not shown). This is consistent with the lack of a synergistic rise in $[Ca^{2+}]_i$ obtained in PMN treated with phosphatidylinositol-specific PLC, which reduces the amount of Fc γ RIIIB on the cell surface by 80% (35 and data not shown).

The ITAM of Fc γ RIIA Is Required for Calcium Flux

Activation of tyrosine phosphorylation and propagation of

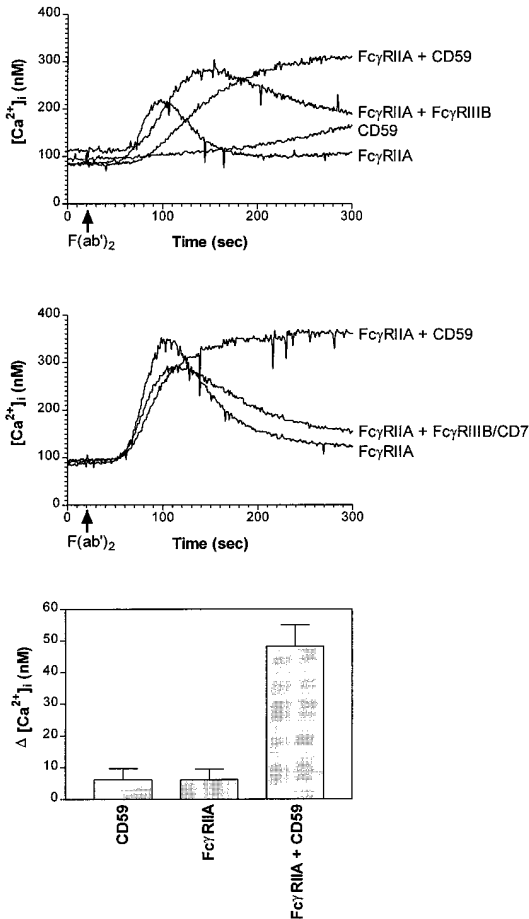


Figure 3. $[Ca^{2+}]_i$ in cells expressing the chimeric Fc γ RIIIB/CD7. J2/3 cells (*top*), J2/3-CD7 cells (*middle*), or PMN (*bottom*) were preloaded with Fura 2-AM. J2/3 and J2/3-CD7 cells were then incubated for 30 min with the mAb IV.3 (anti-Fc γ RII), mAb 3G8 (anti-Fc γ RIIIB), mAb MEM-43 (anti-CD59), or combinations of these mAbs. PMN were incubated with mAb IV.3 F(ab), mAb 10G10 F(ab')₂ (anti-CD59), or combinations of these mAbs. Experiments were performed as described in Fig. 2. Each curve is representative of at least three independent experiments. For PMN, the change in $[Ca^{2+}]_i$ at 140 s after the addition of cross-linking antibody was calculated and results are shown as the mean \pm SEM for three independent experiments (*bottom*).

a tyrosine kinase cascade by receptor associated ITAMs is thought to be essential for Fc γ receptor signaling (16, 43). To determine whether this cascade had a role in Fc γ receptor synergy, Jurkat cells were transfected with Fc γ RIIIB and a mutant Fc γ RIIA in which tyrosines Y²⁸² and Y²⁹⁸ contained within the ITAM were mutated to phenylalanines (J2Y \rightarrow F/3; Fig. 1, *bottom*). It has been shown in model systems that these tyrosines are required for $[Ca^{2+}]_i$ flux when Fc γ RIIA is ligated alone (27, 28). No synergistic $[Ca^{2+}]_i$ flux occurred in J2Y \rightarrow F/3 cells when Fc γ RIIA was ligated either alone or together with Fc γ RIIIB, although these cells were fully competent to increase $[Ca^{2+}]_i$ in response to antigen receptor ligation (Fig. 4). Therefore, these tyrosines in the cytoplasmic tail of Fc γ RIIA are required for the synergistic $[Ca^{2+}]_i$ rise. Thus both the GPI anchor of Fc γ RIIIB and the ITAM motif of Fc γ RIIA are required for synergy in calcium signaling.

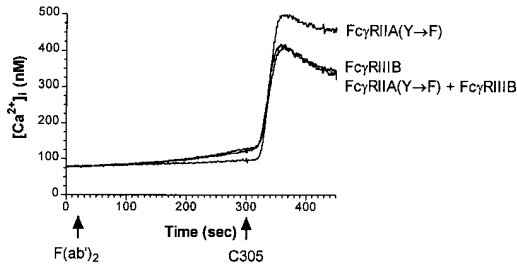


Figure 4. $[Ca^{2+}]_i$ flux in cells expressing Fc γ RIIA containing the ITAM mutation. Fura 2-AM preloaded J2Y \rightarrow F/3 cells were incubated with the mAbs IV.3 (anti-Fc γ RII) and 3G8 (anti-Fc γ RIIIB), then analyzed by fluorimetry as described in Fig. 2. The mAb C305, specific for the TCR/CD3 complex, was added at 300 sec to demonstrate that these cells are competent to flux $[Ca^{2+}]_i$.

The Synergistic Signal Does Not Result in Increased Tyrosine Phosphorylation of Fc γ RIIA

Because of the requirement for the ITAM in synergy and the association of GPI-linked proteins with src family kinases (4, 43), we hypothesized that an early step in this synergistic interaction might be an increased tyrosine phosphorylation of the ITAM of Fc γ RIIA. When Fc γ RIIA was immunoprecipitated from J2/3 cells after crosslinking Fc γ RIIA alone, its tyrosine phosphorylation peaked at 1 min and was diminished by 5 min (Fig. 5 A, *top*). Surprisingly, crosslinking Fc γ RIIA and Fc γ RIIIB together did not enhance tyrosine phosphorylation of Fc γ RIIA as expected but actually diminished detection of the tyrosine phosphorylation of Fc γ RIIA (Fig. 5 A, *top*). Averages from three experiments after normalization for the amount of receptor immunoprecipitated showed that Fc γ RIIA was phosphorylated \sim 10-fold less under synergistic conditions as compared to ligation of Fc γ RIIA alone. We also analyzed the tyrosine phosphorylation of Fc γ RIIA in J2/3-CD7 cells. Ligation of Fc γ RIIA without Fc γ RIIIB induced tyrosine phosphorylation of itself to a similar extent and with similar kinetics as in cells expressing both wild-type Fc γ receptors (Fig. 5 B, *bottom*). In striking contrast to the results obtained in J2/3 cells by crosslinking both wild-type Fc receptors, cocrosslinking Fc γ RIIA and Fc γ RIIIB/CD7 did not significantly diminish the extent or alter the kinetics of Fc γ RIIA phosphorylation (Fig. 5 A, *bottom*). To determine if the marked diminution of Fc γ RIIA tyrosine phosphorylation also occurred when it was crosslinked with other GPI-anchored proteins, Fc γ RIIA was crosslinked with CD48 or CD59 (Fig. 5 B). Cocrosslinking any GPI-anchored protein with Fc γ RIIA markedly diminished its tyrosine phosphorylation. In addition, we analyzed the extent of tyrosine phosphorylation of Fc γ RIIA in PMN after ligating Fc γ RIIA, individually or together with Fc γ RIIIB, by using the F(ab) fragment of mAb IV.3 and the F(ab')₂ of mAb 3G8. Crosslinking both Fc γ receptors resulted in \sim 2–3-fold diminished tyrosine phosphorylation of Fc γ RIIA when compared to ligating Fc γ RIIA alone (data not shown).

The Synergistic Calcium Rise Does Not Result from the Prolonged Tyrosine Phosphorylation of PLC- γ 1

PLC- γ 1 is one of several PLC isoforms that converts phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inosi-

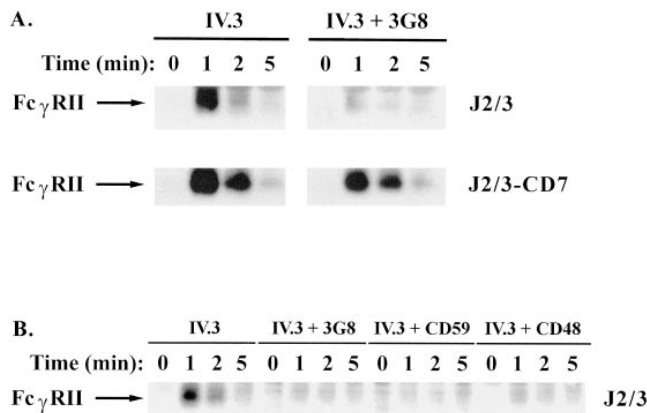


Figure 5. Tyrosine phosphorylation of Fc γ RIIA after crosslinking Fc γ R. (A) J2/3 (top) or J2/3-CD7 (bottom) cells were incubated with mAb IV.3 (anti-Fc γ R2) or with mAbs IV.3 and 3G8 (anti-Fc γ R3) for 30 min on ice and then warmed 10 min to 37°C. (B) J2/3 cells were incubated with various combinations of mAbs specific for Fc γ R2, Fc γ R3, CD48, or CD59. In both panels, crosslinking F(ab')₂ fragments of goat anti-mouse antibodies were added for various amounts of time. At each time point, an aliquot was removed, lysed, and Fc γ RIIA immunoprecipitated. Proteins were separated by SDS-PAGE, and blots were probed with anti-phosphotyrosine. Cocrosslinking of GPI- but not transmembrane-anchored Fc γ RIIB diminishes tyrosine phosphorylation of Fc γ RIIA. Blots shown are representative of at least five experiments.

tol 1,4,5-triphosphate leading to the release of intracellular stores of calcium. In several cell types, crosslinking Fc γ RIIA induces the tyrosine phosphorylation of PLC- γ 1, which leads to its activation (25, 42). To determine whether prolonged activation of PLC- γ 1 could account for the synergistic increase in [Ca²⁺]_i, its tyrosine phosphorylation was examined. In agreement with previous studies, crosslinking Fc γ RIIA in the transfected Jurkat cells resulted in tyrosine phosphorylation of PLC- γ 1 that was visible by 1 min (data not shown, and 42). Crosslinking Fc γ RIIB and Fc γ RIIA in J2/3 cells resulted in tyrosine phosphorylation of PLC- γ 1, which was not different from cocrosslinking Fc γ RIIA and the chimeric Fc γ RIIB/CD7 in J2/3-CD7 cells (Fig. 6). Thus, Fc γ receptor synergy is independent of the tyrosine phosphorylation of PLC- γ 1.

The Synergistic Rise in [Ca²⁺]_i Requires the Influx of Extracellular Calcium

To determine the source of Ca²⁺ for the synergistic [Ca²⁺]_i rise in the J2/3 cells, changes in Fura-2 fluorescence were measured in the presence of extracellular EGTA to prevent calcium influx from the medium. The synergistic [Ca²⁺]_i rise was inhibited almost immediately after addition of EGTA, indicating that calcium influx through plasma membrane channels is largely responsible for the prolonged [Ca²⁺]_i rise (Fig. 7 A, left) as found in PMN (44). Similarly, the synergistic [Ca²⁺]_i rise induced by cocrosslinking Fc γ RIIA and CD59 was abolished by the addition of EGTA (Fig. 7 A, middle). As a control, the changes in intracellular calcium were measured after the T-cell receptor complex (TCR/CD3) was crosslinked with the mAb C305 (Fig. 7 A, right). Previous studies have shown that

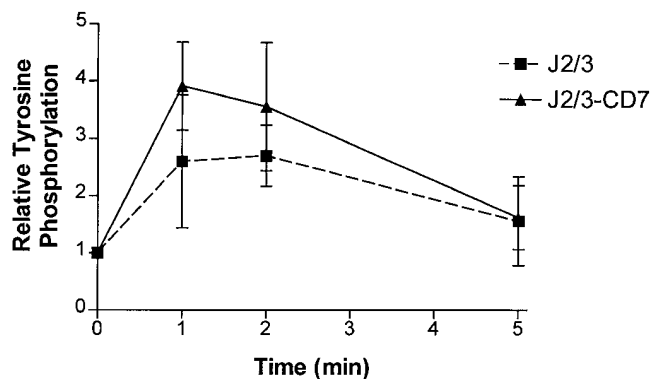


Figure 6. The tyrosine phosphorylation of PLC- γ 1 after crosslinking various Fc γ R. J2/3 (squares) or J2/3-CD7 (triangles) cells were incubated with mAbs IV.3 (anti-Fc γ R2) and 3G8 (anti-Fc γ R3), warmed to 37°C, and crosslinking initiated by addition of F(ab')₂ fragments of goat anti-mouse antibodies. At each time point, an aliquot was removed, PLC- γ 1 was immunoprecipitated, and proteins were separated by SDS-PAGE. Blots were probed with anti-phosphotyrosine and subsequently with anti-PLC- γ 1 antibodies to determine the relative phosphorylation of the immunoprecipitated enzyme, as described in Materials and Methods. Three independent experiments from both cell types were analyzed by densitometry, and the mean and SEM of the three experiments are shown.

the rise in intracellular calcium after TCR crosslinking results from an initial rise derived from intracellular stores followed by a secondary sustained calcium influx through plasma membrane channels that can be abolished by the addition of EGTA (41). The addition of EGTA to Jurkat cells treated only with crosslinking secondary antibody does cause a small decrease in the amount of intracellular calcium, but this small depletion does not account for the large loss in the synergistic calcium influx from extracellular stores, as previously shown in PMN (37; Fig. 7, A and C, left). The changes in intracellular calcium also were measured when EGTA was added immediately before Fc γ receptor crosslinking (Fig. 7 B, left). Crosslinking led to an initial rise in [Ca²⁺]_i, but the synergistic [Ca²⁺]_i rise was substantially diminished after cocrosslinking Fc γ RIIA with Fc γ RIIB or CD59 (Fig. 7 B, middle and right). The magnitude of the [Ca²⁺]_i rise also was diminished in the presence of EGTA, again demonstrating that a significant contribution to the [Ca²⁺]_i rise is due to the influx of extracellular calcium (Fig. 7 B). The slow rise in [Ca²⁺]_i after crosslinking either Fc γ RIIB or CD59 alone was abolished in the presence of EGTA (Fig. 7 C, right, and data not shown). EGTA treated cells do not produce a flux in [Ca²⁺]_i after the addition of crosslinking secondary antibodies alone (Fig. 7 C, left).

Discussion

Since the discovery that GPI-linked proteins can transduce proliferative signals, attention has focused on the mechanism by which these proteins, anchored into the outer leaflet of the plasma membrane by their fatty acyl chains, can signal to the cell cytoplasm. Two distinct but not mutually exclusive paradigms have developed. One model suggests that GPI-linked proteins can sequester into specialized

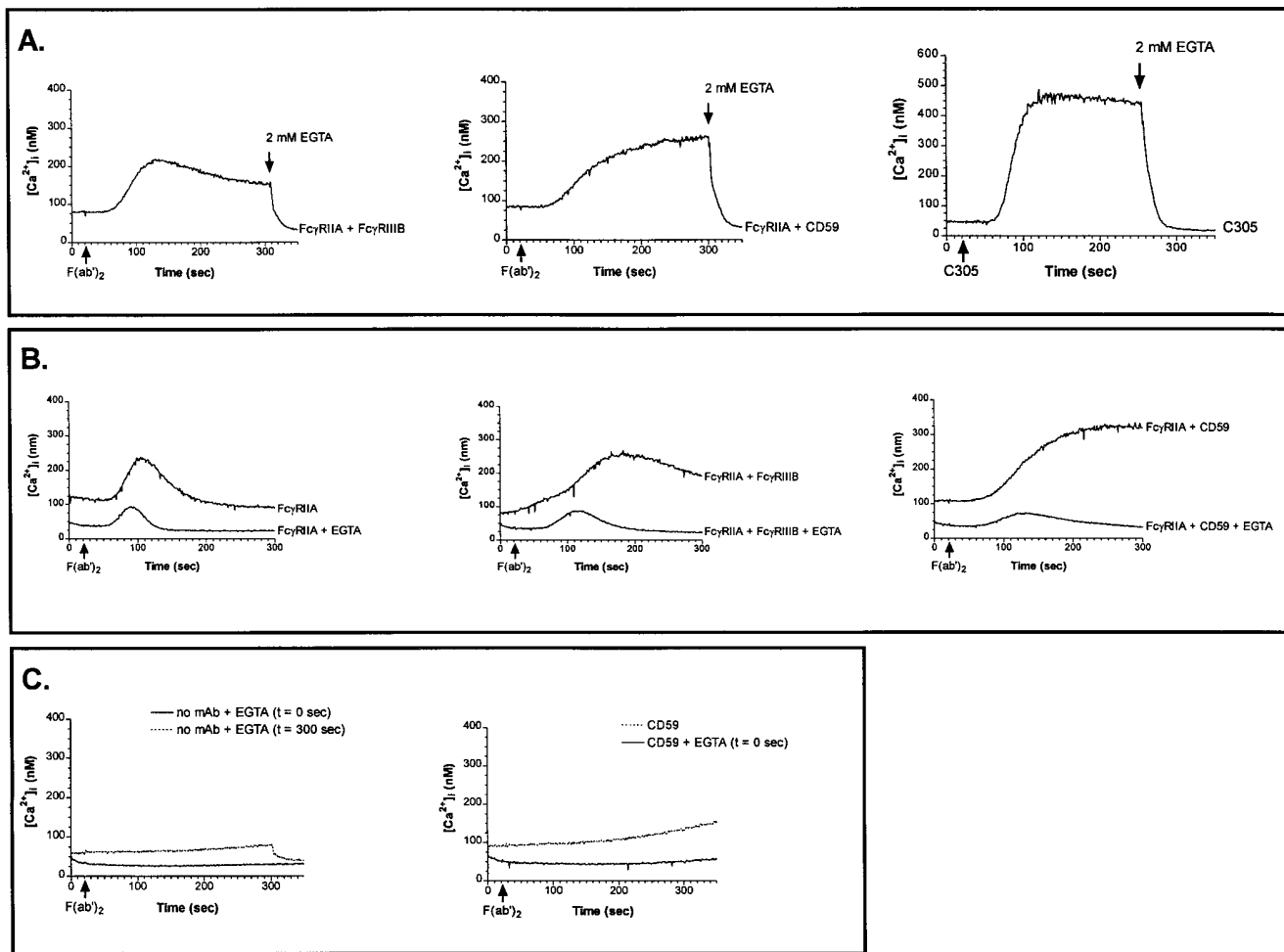


Figure 7. The synergistic rise in $[Ca^{2+}]_i$ requires the influx of extracellular calcium. Changes in Fura 2-AM fluorescence after receptor crosslinking in J2/3 cells was measured as in Fig. 2 in the absence or presence of 2 mM EGTA to prevent calcium influx from the medium. (A) 2 mM EGTA was added 280 s after crosslinking. (B) 2 mM EGTA was added immediately before receptor crosslinking. Also shown is no added EGTA. (C) 2 mM EGTA was added at 0 or 300 s.

membrane domains, especially after clustering (for review see 29, 34). These domains, which are defined by their insolubility in Triton X-100, contain characteristic lipid components, such as glycosphingolipids and cholesterol, but may be depleted in certain phospholipids. GPI-linked proteins are enriched ~200-fold in these domains, and there is evidence for concentration of Src kinases, G protein-coupled receptors, and heterotrimeric G proteins in these membrane domains as well. This has led some investigators to hypothesize that these domains function in signal transduction, and indeed crosslinking of GPI-linked proteins leads to rapid induction of tyrosine phosphorylation (43). On the other hand, some src family kinases sequestered in these domains have low specific activity, suggesting that these glycolipid domains function not in signaling but as a reservoir of signaling molecules that can be recruited to other parts of the membrane (34).

The second model for signal transduction by GPI-linked proteins involves their physical association with transmembrane proteins. For example, Fc γ RIIIB has been shown to associate with the integrin Mac-1, as has the GPI-linked urokinase receptor (uPAR), which also can associate with

another integrin, $\alpha v\beta 3$ (21, 46). These physical associations have functional consequences, for example, induction of IgG-mediated phagocytosis in transfected 3T3 cells (21), or cellular adhesion to vitronectin (46). Thus, it is possible that GPI-linked proteins transduce information to the cytoplasm through physical interaction with transmembrane proteins.

The interaction of Fc γ RIIA and Fc γ RIIIB on human PMN presents an opportunity to test these hypotheses concerning signal transduction by GPI-linked proteins. When immune complexes bind to PMN, Fc γ RIIA and Fc γ RIIIB are brought into proximity. While synergy between the receptors in signal transduction in response to immune complexes has been shown, interpretation is complicated by the interaction of both receptors with other membrane proteins such as Mac-1 (40, 48), and by the inability to use molecular genetic techniques to probe receptor function in these primary cells. For these reasons, we have developed a model system to understand Fc γ receptor synergy on PMN. In Jurkat cells without Mac-1, Fc γ RIIA and Fc γ RIIIB can synergize to increase $[Ca^{2+}]_i$, demonstrating that extracellular domain association with Mac-1

is not required for at least this aspect of synergy. Indeed, since coligation of two other GPI-linked proteins, otherwise structurally unrelated to Fc γ RIIIB, also can synergize with Fc γ RIIA to increase $[Ca^{2+}]_i$, it is unlikely that extracellular domain interactions other than with multivalent ligands are required to induce synergy between the transmembrane and GPI-linked Fc γ receptors. The synergistic increase in $[Ca^{2+}]_i$ may be important in numerous PMN functions, including degranulation (3, 23), actin polymerization (2), and phagocytosis (17, 18).

Our data support the hypothesis that association of Fc γ RIIA with glycolipid domains enriched in GPI-linked proteins fundamentally alters subsequent signaling. Crosslinking Fc γ RIIA with any of the GPI-linked proteins induced the synergistic increase in $[Ca^{2+}]_i$ and, surprisingly, decreased the extent of Fc γ RIIA tyrosine phosphorylation. When Fc γ RIIIB was expressed with a transmembrane domain, its synergy with Fc γ RIIA was abolished, as was its effect on Fc γ RIIA tyrosine phosphorylation. These data support the hypothesis that the membrane environment of Fc γ RIIA is altered by crosslinking it with GPI-anchored proteins. This altered environment modulates the Fc γ RIIA-generated signal in fundamental ways. We initially expected that the synergistic $[Ca^{2+}]_i$ rise would be associated with increased phosphorylation of the ITAM of Fc γ RIIA, because src family kinases, which phosphorylate ITAMs, have been found to be concentrated in these domains. However, our finding of decreased tyrosine phosphorylation is consistent with the report that CD45, the major transmembrane tyrosine phosphatase present on lymphocytes, is excluded from glycolipid-enriched membrane domains, resulting in lower specific activity of the lymphocyte src kinases in these domains (34). We propose that Fc γ RIIA has diminished tyrosine phosphorylation after cocrosslinking with Fc γ RIIIB, because ligation with GPI-linked proteins causes Fc γ RIIA to be brought into membrane domains with less-active src kinases. It is also possible that an additional signaling pathway is used to mediate synergistic calcium signaling, since the prolonged rise in intracellular calcium is not due to the prolonged tyrosine phosphorylation of PLC- γ 1. Calcium mobilization after crosslinking Fc ϵ RI activates a sphingosine kinase that produces sphingosine-1-phosphate as a second messenger for intracellular calcium mobilization (6). Alternatively, localization of the Fc γ receptors within specialized membrane domains may activate the synergistic influx of extracellular calcium. Indeed, a plasma membrane calcium pump has been identified in caveolae (10).

Our data further extend the observations made with several receptors, including Fc γ receptors, that there may be interaction on the cell surface between receptors recognizing the same ligand. For example, T cells express two distinct receptors that interact with MHC class I molecules, one that mediates the positive signal, the T cell receptor, and a second receptor, NKB1, that mediates an inhibitory signal (22, 31). It has been observed in phagocytic cells that the Fc γ receptor, Fc γ RIIB, inhibits phagocytosis mediated by Fc γ RIIA. Decreased tyrosine phosphorylation induced by Fc γ RIIB after interaction with IgG ligand may be responsible for this inhibition of Fc γ RIIA-mediated phagocytosis (Hunter, S., and A.D. Schreiber, unpublished results).

In summary, transfection of human PMN Fc γ receptors into the Jurkat cell line has allowed for the further dissection of the mechanism by which these receptors cooperate in immune complex-induced PMN activation. We have defined two essential structural components of the synergistic signal, the GPI-anchor of Fc γ RIIIB and the ITAM of Fc γ RIIA. Moreover, we have shown that synergy can occur in the absence of the phagocyte integrin Mac-1, previously postulated to be an essential component for synergy. In PMN, 10,000 to 20,000 Fc γ RIIA molecules are expressed on the cell surface together with 10 to 20 times more Fc γ RIIIB (12, 13). Thus it is highly likely that whenever Fc γ RIIA is ligated by an immune complex, it is in association with several GPI-linked Fc γ RIIIB and that the modulated signal which occurs because of association with GPI domains is the major mechanism of immune complex-mediated PMN activation.

We thank Dr. Ming-jie Zhou (Molecular Probes, Inc.) for the PCR clone of CD16, Dr. Brian Seed for the CD16/CD7/ ζ cDNA, Dr. Andrew Chan for the C305 mAb, Dr. Jurgen Frey for the IIIA5 mAb, and Drs. Doug Lublin and Scott Blystone (Washington University, St. Louis, MO) for helpful discussions.

This work was supported by grants from the National Institutes of Health and the Arthritis Foundation to E.J. Brown. J.M. Green is supported as a Lucille P. Markey Pathway postdoctoral fellow.

Received for publication 29 April 1997 and in revised form 13 August 1997.

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