Role for a Glycan Phosphoinositol Anchor in Fcγ Receptor Synergy

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Abstract. While many cell types express receptors for the Fc domain of IgG (FcγR), only primate polymorphonuclear neutrophils (PMN) express an FcγR linked to the membrane via a glycan phosphoinositol (GPI) anchor. Previous studies have demonstrated that this GPI-linked FcγR (FcγRIIIB) cooperates with the transmembrane FcγR (FcγRIIA) to mediate many of the functional effects of immune complex binding. To determine the role of the GPI anchor in Fcγ receptor synergy, we have developed a model system in Jurkat T cells, which lack endogenously expressed Fcγ receptors. Jurkat T cells were stably transfected with cDNA encoding FcγRIIA and/or FcγRIIIB. Cocrosslinking the two receptors produced a synergistic rise in intracellular calcium ([Ca2+]i) to levels not reached by stimulation of either FcγRIIA or FcγRIIIB alone. Synergy was achieved by prolonged entry of extracellular Ca2+. Cocrosslinking FcγRIIA 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 FcγRIIA on PMN, suggesting that interactions between the extracellular domains of the two Fcγ receptors are not required for synergy. Replacement of the GPI anchor of FcγRIIIB with a transmembrane anchor abolished synergy. In addition, tyrosine to phenylalanine substitutions in the immunoreceptor tyrosine-based activation motif (ITAM) of the FcγRIIA cytoplasmic tail abolished synergy. While the ITAM of FcγRIIIB was required for the increase in [Ca2+]i, tyrosine phosphorylation of crosslinked FcγRIIIB was diminished when cocrosslinked with FcγRIIIB. These data demonstrate that FcγRIIA association with GPI-linked proteins facilitates FcγR signal transduction and suggest that this may be a physiologically significant role for the unusual GPI-anchored FcγR of human PMN.

The binding of immune complexes by polymorphonuclear neutrophils (PMN) stimulates many cell types expressing receptors for the Fc domain of IgG (FcγR) to produce a synergistic rise in intracellular calcium ([Ca2+]i) and intracellular signaling events, such as the activation of the src family member hck and induction of intracellular signaling pathways. These studies, taken together with this receptor’s role in the immune response, suggest that the unusual GPI-anchored FcγR may play a physiological role in mediating immune complex presentation to FcγRIIA (1, 3), which is the only known FcγR expressed on human PMN. The phosphorylated ITAM of FcγRIIA 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 FcγRIIB, the most abundant PMN FcγR. Some studies have suggested an inability of FcγRIIB to transduce signals independently. These studies, taken together with this receptor’s lack of a cytoplasmic domain, have led to the concept that FcγRIIB is primarily an Fc-binding molecule that aids in immune complex presentation to FcγRIIA (1, 3). However, evidence now suggests that FcγRIIB is able to mediate intracellular signaling events, such as the activation of the src family member hck and induction of intracellular signaling pathways.

[Ca2+]i, intracellular calcium concentration; GPI, glycan phosphoinositol; ITAM, immunoreceptor tyrosine-based activation motif; PLC, phospholipase C; PMN, polymorphonuclear neutrophils.

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

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The Journal of Cell Biology, Volume 139, Number 5, December 1, 1997 1209–1217
http://www.jcb.org
conditions that led to the synergistic calcium flux and that tyrosine kinase signaling. In transfected Jurkat T cells, the rise required the influx of extracellular calcium and depended upon the GPI anchor of FcRIIA and FcRIIB in Jurkat T cells, which lack endogenous Fc receptors but are fully competent for tyrosine kinase signaling. In transfected Jurkat T cells, the PMN Fc receptors synergized to induce a rise in intracellular calcium concentration ([Ca^{2+}]_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 FcRIIB, since a mutant in which the GPI anchor was replaced by the transmembrane domain of CD7 was unable to synergize with FcRIIA. Moreover, crossinglinking other GPI-linked proteins on Jurkat T cells with FcRIIA also led to a synergistic increase in [Ca^{2+}]_i. The increase in [Ca^{2+}]_i, also required the tyrosines of the FcRIIA 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-γ1 phosphorylation was not altered by the replacement of the GPI anchor of FcRIIB with the transmembrane domain of CD7. Thus, synergy between FcγR requires the GPI anchor of FcRIIB, but not for an increase in FcγRIIA-dependent tyrosine kinase signaling. We hypothesize instead that the role for the GPI anchor of FcγRIIB is to sequester FcγRIA 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 FcγR 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 t-glutamine, 0.1 mM NEAA, 50 mM 2-mercaptoethanol, and 100 μg/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-FcγRII); 26g, 3G8 (anti-CD16, anti-FcγRII), 9I4 (anti-CD55, anti-DAF); 8M, 44-M3 (anti-CD38, anti-Proteinase 3); 10G10 (anti-CD32) kindly provided by Dr. Marilyn Telen, Duke University, Durham, NC); MEM-102 (anti-CD48; Harlan Bioproducts, Indianapolis, IN), 11A5 (anti-FcγRII; kindly provided by Dr. Jurgen Frey, Universität Bielefeld), and mouse IgG2a 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 IgG₂ (Sigma Chemical Co.) were used. Antibody fragments of IV.3, 3G8, or 10G10 were made by standard meth- odologies (Medarex, Inc., Madison, NJ). For bound mAbs were detected using FITC-conjugated goat F(ab′)₂, fragments specific for mouse F(ab′) (Sigma Chemical Co.). Anti-phospholipase C-γ1 (PLC-γ1) 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 IgG₁ (Caltag Laboratories, San Francisco, CA).

**FcγRIIA and FcγRIIB Expression Constructs and Transfection into Jurkat T Cells**

The oligos 5'-CCTGAATTCCTCGGATATCTTGGTGAC-3' and 5'-AGAGGATCTCGCATGCTTATAC-3' were used to amplify the human FcγRIIB (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−/+ or pRcCMV, and pCPE4 (Invitrogen, San Diego CA). The intactness of the cDNA was verified by DNA sequencing (ABI PRISM Dye Terminator Cycle Sequence Ready Reaction Kit; Perkin Elmer, Foster City, CA). The FcγRIIB/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) and a MluI/NotI adapter (annealed oligonucleotides 5'-CGGCCCTAAATAGCATGATGCC-3' and 5'-GCCCCTACTGATCTTAAA-3' stop codons underlined) into HindIII/NotI-digested pRcCMV. This construct encodes the FcγRIIB extracellular domain joined with a CD7 transmembrane domain. The cDNA was verified by DNA sequencing. The cDNAs encoding FcγRIIA and FcγRIIB with both ITAM tyrosines in the cytoplasmic tail mutated to phenylalanine were prepared as described (7, 27) and cloned into pRcCMV and pCPE4.

The resulting plasmids were introduced into clones of Jurkat T cells by electroporation. Cells (10⁷) in 400 μl HEBS (25 mM Hepes, pH 7.05, 140 mM NaCl, 750 mM Na₂HPO₄) and plasmid (30 μg in 100 μl HEBS) were added to a 0.4-mm-gap width cuvette and electroporated at 1,000 V/cm 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 μg/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 (10⁷) were resuspended in 50 μl PBS/5% FCS with 1 μg 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.

**[Ca^{2+}]_i Measurements**

Jurkat transfectants were loaded with 3 μM 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 μM 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 × 10⁷) were then 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 TdR, 50 μg/ml BSA, 0.5 mM MgCl₂, 0.5 mM EGTA) in 100 μl HBSS++ for 30 min to wash out bound mAbs. Cells (1 × 10⁷) were loaded with 10 μM Fura 2-AM in HBSS++ at 37°C, washed, and resuspended at 10⁶ cells/ml in HBSS++. 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 μM mouse F(ab′)₂ 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 × 10⁷) were incubated in RPMI 1640/10% FCS containing the mAb IV.3 (15 μg/ml) or the mAbs IV.3 and 3G8 (15 μg/ml each) for 30 min.
Results

Cocrosslinking FcγRIIA and FcγRIIIB Results in a Synergistic [Ca^{2+}]_i Rise

Jurkat T cells, which do not express endogenous Fc receptors, were stably transfected with the cDNAs encoding FcγRIIA and FcγRIIIB (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 FcγRIIIB 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 FcγRIIIB 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 [Ca^{2+}]_i in J2/3 cells after crosslinking FcγRIIA and FcγRIIIB individually or after crosslinking both receptors together, using a F(ab')_2 crosslinking antibody. Crosslinking FcγRIIA resulted in a significant, short lived rise in [Ca^{2+}]_i (Fig. 2, top). In contrast, crosslinking FcγRIIIB alone resulted in a slow rise in [Ca^{2+}]_i with a magnitude lower than for FcγRIIA (Fig. 2, top). When both FcγR were crosslinked together, there was an increase in the maximum [Ca^{2+}]_i rise and a prolongation of the increase (Fig. 2, top). Synergy did not require the Fc fragment of either anti-FcγRII or FcγRII 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 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 [Ca^{2+}]_i (Fig. 2, top and data not shown). In PMN, cocrosslinking FcγRIIIB 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 FcγRIIIB expression. In PMN, FcγRIIIB 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 FcγRIIIB from the cell surface, abolishes the rise in [Ca^{2+}]_i after FcγRIIIB crosslinking (35, and data not shown). Nonetheless, the expression level of FcγRIIIB in the transfeected Jurkat cells was sufficient to produce a synergistic rise in [Ca^{2+}]_i.

To determine if the synergistic calcium response required bridging of FcγRIIA and FcγRIIIB together or whether the augmentation in [Ca^{2+}]_i could be achieved by simultaneously crosslinking each Fc receptor individually, isoype-specific secondary crosslinking antibodies were used (Fig. 2, middle). FcγRIIA was crosslinked with IV.3, an IgG2b mAb, and goat F(ab')_2 fragments specific for mouse IgG2b, and FcγRIIIB 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 [Ca^{2+}]_i, was found (Fig. 2, middle), paralleling results found in PMN (44). In fact, the resulting rise in [Ca^{2+}]_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

min on ice. Cells were washed three times, resuspended in 0.5 ml RPMI 1640 with 10% FCS, and then warmed to 37°C for 10 min. Crosslinking mouse F(ab') specific goat F(ab'); 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 NαVO₄, 1 mM Na₃MoO₄, 1 mM Na₂H₂P₂O₇, 10 ng/ml calyculin, 25 μg/ml aprotonin, 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, bromphenol blue). Samples were boiled for 5 min and then subjected to SDS-PAGE and electrotransfer onto Immobilon-P (Millipore, Bedford, MA) membranes. Blots were probed with anti-phosphotyrosine, anti-FcγRII (II1A5), 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.

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 FcγRIIIB, 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 FcγRIIIB (J2/3; top), wild-type FcγRIIA and the chimeric FcγRIIIB/CD7 (J2/3-CD7; middle), or wild-type FcγRIIIB and the mutant FcγRIIA where the tyrosines within the ITAM (Y282 and Y298) are changed to phenylalanine (J2Y→F/3; bottom) are shown.
The GPI Anchor Is Necessary and Sufficient for the Contribution of FcyRIIIB to Synergy

Primate PMN are the only cells that express a GPI-anchored Fcy receptor (32). To determine whether the GPI anchor was necessary for FcyRIIIB contribution to the synergistic increase in \([\text{Ca}^{2+}]_i\), stable transfectants were made expressing FcyRIIA and a chimeric FcyRIIIB with the GPI anchor replaced by the transmembrane domain of CD7 (J2/3-CD7; Fig. 1, middle). When FcyRIIA and FcyRIIIB/CD7 were crosslinked together in these cells, the \([\text{Ca}^{2+}]_i\) rise was similar to the rise generated when FcyRIIA was crosslinked alone without any synergy from FcyRIIIB (Fig. 3, middle). The inability of the chimeric FcyRIIIB/CD7 molecule to contribute to the synergistic \([\text{Ca}^{2+}]_i\) rise was not due to inadequate expression of this protein, since the FcyRIIIB/CD7 molecule was expressed at a greater level than the wild-type FcyRIIIB (Fig. 1, top and middle). This experiment demonstrates that the GPI anchor is necessary for the synergistic \([\text{Ca}^{2+}]_i\) rise.

To determine whether any aspect of the extracellular Ig domains of FcyRIIIB rise were required for the synergistic \([\text{Ca}^{2+}]_i\) rise, other GPI-linked proteins expressed by Jurkat cells were cocrosslinked with FcyRIIA. 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 FcyRIIIB. When these GPI-linked proteins, CD59 (Fig. 3, top) and CD48 (not shown), were cocrosslinked with FcyRIIA, a synergistic rise in \([\text{Ca}^{2+}]_i\) also occurred in Jurkat cells transfected with FcyRIIIB 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 \([\text{Ca}^{2+}]_i\) rise similar to that elicited by crosslinking FcyRIIIB alone (Fig. 3, top, and data not shown).

These experiments demonstrate that the GPI anchor of FcyRIIIB is required for FcyR cooperation but that other extracellular domains will substitute for FcyRIIIB when cocrosslinked with FcyRIIA. 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 FcyRIIA and FcyRIIIB interaction with the \(b\) integrin CR3 (for review see 30). Moreover, since these cells do not express CR3, this experiment shows that FcyR synergy can occur without this PMN integrin.

Synergy in PMN between FcyRIIA and FcyRIIIB was found for the rise in \([\text{Ca}^{2+}]_i\) (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 \([\text{Ca}^{2+}]_i\), could also be obtained in PMN with other GPI-anchored proteins, FcyRIIA and CD59 were cocrosslinked and a prolongation in the rise \([\text{Ca}^{2+}]_i\) was found (Fig. 3, bottom). The synergistic rise in \([\text{Ca}^{2+}]_i\), with FcyRIIA and CD59 was not as pronounced as with FcyRIIIB and FcyRIIA. No significant synergy between FcyRIIA 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 FcyRIIIB (CD59 has \(~13\)% of the expression of FcyRIIIB, CD48 has 1%, CD55 has 6%, and CD66b has 9%; data not shown). This is consistent with the lack of a synergistic rise in \([\text{Ca}^{2+}]_i\), obtained in PMN treated with phosphatidylinositol-specific PLC, which reduces the amount of FcyRIIIB on the cell surface by 80% (35 and data not shown).

The ITAM of FcyRIIA Is Required for Calcium Flux

Activation of tyrosine phosphorylation and propagation of
a tyrosine kinase cascade by receptor associated ITAMs is thought to be essential for Fcγ receptor signaling (16, 43). To determine whether this cascade had a role in Fcγ receptor synergy, Jurkat cells were transfected with FcγRIIB and a mutant FcγRIIA in which tyrosines Y282 and Y298 contained within the ITAM were mutated to phenylalanines (J2Y→F/3; Fig. 1, bottom). It has been shown in model systems that these tyrosines are required for [Ca^{2+}]_i flux when FcγRIIA is ligated alone (27, 28). No synergistic [Ca^{2+}]_i flux occurred in J2Y→F/3 cells when FcγRIIA was ligated either alone or together with FcγRIIB, 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 FcγRIIA are required for the synergistic [Ca^{2+}]_i rise. Thus both the GPI anchor of FcγRIIB and the ITAM motif of FcγRIIA are required for synergy in calcium signaling.

**The Synergistic Signal Does Not Result in Increased Tyrosine Phosphorylation of FcγRIIA**

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

**The Synergistic Calcium Rise Does Not Result from the Prolonged Tyrosine Phosphorylation of PLC-γ1**

PLC-γ1 is one of several PLC isoforms that converts phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inosi-
Fig. 6. The tyrosine phosphorylation of PLC-γ1 after crosslinking various FcyR. J2/3 (squares) or J2/3-CD7 (triangles) cells were incubated with mAbs IV.3 (anti-FcyRII) and 3G8 (anti-FcyRIII), warmed to 37°C, and crosslinking initiated by addition of Fab'2 fragments of goat anti–mouse antibodies. At each time point, an aliquot was removed, PLC-γ1 was immunoprecipitated, and proteins were separated by SDS-PAGE. Blots were probed with anti-phosphotyrosine and subsequently with anti–PLC-γ1 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.

denot 1,4,5-triphosphate leading to the release of intracellular stores of calcium. In several cell types, crosslinking FcyRIIA induces the tyrosine phosphorylation of PLC-γ1, which leads to its activation (25, 42). To determine whether prolonged activation of PLC-γ1 could account for the synergistic increase in [Ca\(^{2+}\)], its tyrosine phosphorylation was examined. In agreement with previous studies, crosslinking FcyRIIA in the transfected Jurkat cells resulted in tyrosine phosphorylation of PLC-γ1 that was visible by 1 min (data not shown, and 42). Crosslinking FcyRIIB and FcyRIIA in J2/3 cells resulted in tyrosine phosphorylation of PLC-γ1, which was not different from cocrosslinking FcyRIIA and the chimeric FcyRIIB/CD7 in J2/3-CD7 cells (Fig. 6). Thus, Fcy receptor synergy is independent of the tyrosine phosphorylation of PLC-γ1.

The Synergistic Rise in [Ca\(^{2+}\)], Requires the Influx of Extracellular Calcium

To determine the source of Ca\(^{2+}\) for the synergistic [Ca\(^{2+}\)], 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 [Ca\(^{2+}\)], rise was inhibited almost immediately after addition of EGTA, indicating that calcium influx through plasma membrane channels is largely responsible for the prolonged [Ca\(^{2+}\)], rise (Fig. 7 A, left) as found in PMN (44). Similarly, the synergistic [Ca\(^{2+}\)], rise induced by cocrosslinking FcyRIIA 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 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 Fcy receptor crosslinking (Fig. 7 B, left). Crosslinking led to an initial rise in [Ca\(^{2+}\)], but the synergistic [Ca\(^{2+}\)], rise was substantially diminished after cocrosslinking FcyRIIA with FcyRIIB or CD59 (Fig. 7 B, middle and right). The magnitude of the [Ca\(^{2+}\)], rise also was diminished in the presence of EGTA, again demonstrating that a significant contribution to the [Ca\(^{2+}\)], rise is due to the influx of extracellular calcium (Fig. 7 B). The slow rise in [Ca\(^{2+}\)], after crosslinking either FcyRIIB 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 [Ca\(^{2+}\)], 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...
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, FcγRIIB 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, αvβ3 (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 FcγRIIA and FcγRIIB on human PMN presents an opportunity to test these hypotheses concerning signal transduction by GPI-linked proteins. When immune complexes bind to PMN, FcγRIIA and FcγRIIB 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 Fcγ receptor synergy on PMN. In Jurkat cells without Mac-1, FcγRIIA and FcγRIIB can synergize to increase [Ca^{2+}]_i, demonstrating that extracellular domain association with Mac-1

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.
is not required for at least this aspect of synergy. Indeed, since coligation of two other GPI-linked proteins, otherwise structurally unrelated to FcγRIIB, also can synergize with FcγRIIA to increase \([Ca^{2+}]\), it is unlikely that extracellular domain interactions other than with multivalent ligands are required to induce synergy between the transmembrane and GPI-linked Fcγ receptors. The synergistic increase in \([Ca^{2+}]\) 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 FcγRIIA with glycolipid domains enriched in GPI-linked proteins fundamentally alters subsequent signaling. Co-crosslinking FcγRIIA with any of the GPI-linked proteins induced the synergistic increase in \([Ca^{2+}]\), and, surprisingly, decreased the extent of FcγRIIA tyrosine phosphorylation. When FcγRIIB was expressed with a transmembrane domain, its synergy with FcγRIIA was abolished, as was its effect on FcγRIIA tyrosine phosphorylation. These data support the hypothesis that the membrane environment of FcγRIIA is altered by crosslinking it with GPI-anchored proteins. This altered environment modulates the FcγRIIA-generated signal in fundamental ways. We initially expected that the synergistic \([Ca^{2+}]\) rise would be associated with increased phosphorylation of the ITAM of FcγRIIA, 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 FcγRIIA has diminished tyrosine phosphorylation after cocrosslinking with FcγRIIB, because ligation with GPI-linked proteins causes FcγRIIA to be brought into membrane domains with less-active src kinases. It is also possible that an additional signaling pathway is used to mediate synergetic calcium signaling, since the prolonged rise in intracellular calcium is not due to the prolonged tyrosine phosphorylation of PLC-γ1. Calcium mobilization after crosslinking FceRI activates a sphingosine kinase that produces sphingosine-1-phosphate as a second messenger for intracellular calcium mobilization (6). Alternatively, localization of the Fcγ receptors within specialized membrane domains may activate the synergetic 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 Fcγ 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, NK1.1, that mediates an inhibitory signal (22, 31). It has been observed in phagocytic cells that the Fcγ receptor, FcγRIIB, inhibits phagocytosis mediated by FcγRIIA. Decreased tyrosine phosphorylation induced by FcγRIIB after interaction with IgG ligand may be responsible for this inhibition of FcγRIIA-mediated phagocytosis (Hunter, S., and A.D. Schreiber, unpublished results).

In summary, transfection of human PMN Fcγ 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 synergetic signal, the GPI-anchor of FcγRIIB and the ITAM of FcγRIIA. 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 FcγRIIA molecules are expressed on the cell surface together with 10 to 20 times more FcγRIIB (12, 13). Thus it is highly likely that whenever FcγRIIA is ligated by an immune complex, it is in association with several GPI-linked FcγRIIB 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/CD16ζ CDNA, Dr. Andrew Chan for the C305 mAb, Dr. Jurgen Frey for the II.1.A5 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.

References


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