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*J Immunol* 2000; 164:350-360; doi: 10.4049/jimmunol.164.1.350

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Convergence of Fcγ Receptor IIA and Fcγ Receptor IIB Signaling Pathways in Human Neutrophils

Frank Y. S. Chuang, a,† Massimo Sassaroli, b,† and Jay C. Unkeless c,‡

Human neutrophils (PMNs) express two receptors for the Fc domain of IgG: the transmembrane FcγRIIA, whose cytosolic sequence contains an immunoreceptor tyrosine-based activation motif, and the GPI-anchored FcγRIIB. Cross-linking of FcγRIIB induces cell activation, but the mechanism is still uncertain. We have used mAbs to cross-link selectively each of the two receptors and to assess their signaling phenotypes and functional relation. Cross-linking of FcγRIIB induces intracellular Ca2+ release and receptor capping. The Ca2+ response is blocked by wortmannin and by N,N-dimethylsphingosine, inhibitors of phosphatidylinositol 3-kinase and sphingosine kinase, respectively. Identical dose-response curves are obtained for the Ca2+ release stimulated by cross-linking FcγRIIIA, implicating these two enzymes in a common signaling pathway. Wortmannin also inhibits capping of both receptors, but not receptor endocytosis. Fluorescence microscopy in double-labeled PMNs demonstrates that FcγRIIA colocalizes with cross-linked FcγRIIB. The signaling phenotypes of the two receptors diverge only under frustrated phagocytosis conditions, where FcγRIIB bound to substrate-immobilized Ab does not elicit cell spreading. We propose that FcγRIIB signaling is conducted by molecules of FcγRIIA that are recruited to protein/lipid domains induced by clustered FcγRIIB and, thus, are brought into juxtaposition for immunoreceptor tyrosine-based activation motif phosphorylation and activation of PMNs. The Journal of Immunology, 2000, 164: 350–360.

A pplymorphonuclear leukocytes (PMNs a or neutrophils) play a central role in Ab-mediated cellular immunity. Interaction of FcγR on the surface of PMNs with the Fc domains of IgG molecules in immune complexes or on opsonized targets elicits a pleiotropic response, which includes phagocytosis, degranulation, and an oxidative burst. Receptor cross-linking upon binding to multivalent ligands, rather than ligand binding per se, constitutes the critical event leading to intracellular signaling and cell activation. Human PMNs are unique for their constitutive expression of two atypical FcγR isoforms, FcγRIIA (CD32) and FcγRIIB (CD16B). Unlike other multichain Fc receptors, in which Fc binding and signaling domains are segregated to different subunits, the cytosolic sequence of FcγRIIA contains a slightly modified immunoreceptor tyrosine-based activation motif (ITAM) consisting of two YXXL (where X denotes any amino acid) repeats separated by 12 aa (1, 2). Upon cross-linking, the receptors are brought into juxtaposition, and Src family kinases phosphorylate the conserved ITAM tyrosine residues. Phosphorylated ITAMs then function as docking sites for proteins containing tandem Src homology 2 (SH2) domains, such as tyrosine kinases of the Syk family (3) or the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (4), leading to downstream signaling events.

The second FcγR isoform, FcγRIIB, expressed exclusively on human PMNs, is anchored to the plasma membrane via a C-terminal-linked GPI moiety and thus lacks any obvious means of signal transduction upon cross-linking. However, with a 10-fold higher abundance (135,000 vs 10,000 receptors/cell) (5) and a higher affinity for IgG than FcγRIIA, it may play a predominant role in PMN binding of immune complexes.

Because both FcγR isoforms are likely to be engaged by immune complexes, the questions of whether and how the GPI-anchored receptor may complement FcγRIIA function have been subject to debate. Although one view is that FcγRIIB serves merely to enhance immune complex binding for presentation to FcγRIIA, clear evidence supports an active role for the GPI-anchored isoform in signaling and PMN activation. Thus, without FcγRIIA ligation, FcγRIIB cross-linking induces a rise in the intracellular free calcium concentration ([Ca2+]i) and triggers degranulation and the respiratory burst (6, 7). Co-cross-linking of both FcγRs also leads to synergistic enhancement of [Ca2+]i, transients and the phagocytic response (8, 9). In this study we have investigated PMN activation by specific Ab-mediated cross-linking of each of the two FcγRs to compare their signaling phenotypes and to assess their functional relation.

Whereas the essential role of PI3K in FcγR signaling is known, here we show that the PI3K inhibitor wortmannin blocks with identical efficacy the [Ca2+]i, transients elicited by cross-linking FcγRIIB or FcγRIIA. Contradictory evidence exists about the role of phospholipase Ca and the amount of IP3 generated upon FcγR engagement (10–14). Prompted by a report that the [Ca2+]i,
rise upon clustering of FcεRI in a rat mast cell line was mediated by sphinogosine-1-phosphate (SIP), the product of sphinogosine kinase (SK) (15), we have examined whether this pathway is used by FcyRs in PMNs.

The molecular basis for signaling by the GPI-anchored receptor remains unclear. FcyRIIB is just one of a large group of unrelated proteins anchored via GPI to the PMN surface. No common functional theme has been found for this elaborate post-translational modification. In T lymphocytes, cross-linking any GPI-anchored protein was shown to lead to cell activation mediated by the TCR/CD3 complex (16, 17). Several models for signaling by GPI-anchored proteins have been proposed, invoking a role for either the glycosidic or the lipidic components of the GPI moiety (18, 19). Using immunofluorescence microscopy, we have examined the effect that specific Ab-mediated cross-linking of FcyRIIB has on the surface distribution and colocalization of FcyRIIA with aggregated FcyRIIB. In analogy with the “signaling raft” model (19), we propose that aggregation of FcyRIIB leads to signal transduction via formation of protein/lipid domains to which signaling competent molecules, such as FcyRIIA and protein tyrosine kinases, are recruited.

Materials and Methods

Cells

A murine macrophage cell line, P388D1, was transfected with wild-type (wt) or mutant human FcyRIIA CDNA as previously described (20). Cells transfected with wt (designated PW16) or truncated (D233 and D264) FcyRIIB express 1.1–1.8×10^9 receptors/cell. Human PMNs were isolated from buffy coat (Leukopak) preparations obtained from the Blood Donor Center of the Mount Sinai Hospital. PMNs were collected from the 1.119 g/ml interface of a Histopaque (Sigma, St. Louis, MO) density gradient and washed in DMEM (Sigma) containing 2% heat-inactivated FCS and 20 mM HEPES (pH 7.4). Smaller scale preparations were obtained from whole venous blood by centrifugation using Polymorphprep (Life Technologies, Gaithersburg, MD). PMNs were held at room temperature in incubation buffer (150 mM NaCl, 5 mM KCl, 1 mM CaCl_2, 10 mM glucose, and 10 mM HEPES (pH 7.4)), and the cells were maintained at 37°C for various time intervals. Where indicated, wortmannin (Biomol Research, Plymouth, PA) was also added during this interval. Thereafter, the PMNs were centrifuged onto glass slides (Cytospin 2, Shandon Southern Instruments, Sewickley, PA), promptly fixed in 20°C methanol, air-dried, and mounted in glycerol. Digital images were acquired using a Zeiss Axiosvert microscope (Carl Zeiss, Thornwood, NY), a ×10, 0.25 normal aperture (NA) or a ×40, 0.75 NA objective, a CCD camera (OMA Vision, EG&G PARC, Princeton, NJ), and 560 nm transillumination light selected by a 40-nm bandpass filter (Omega Optical, Brattleboro, VT). Image analysis was performed using Image-1 (Universal Imaging, West Chester, PA).

Labeling of Abs

Anti-FcγRIIB 3G8 mAb was obtained from Rhone Poulenc (Antony, France). The anti-FcγRIIB IV.3 monoclonal cell line was from American Type Culture Collection (Manassas, VA). IV.3 IgG1 and Fab were prepared as previously described (20). Fab were labeled with amine-reactive probes (fluorescein, rhodamine and Texas Red isothiocyanate or succinimidyl esters; Molecular Probes, Eugene, OR) in 0.15 M ethanolamine and removed by passage over a Sephadex G-25 column. Antibody preparations were conjugated to the rabbit anti-FcγRIIB 3G8 Fab (1–5 mg/ml) using sodium iodoacetate (21) or N-succinimidyl 3-(2-pyridyldithio) propionate (22). Each antibody was conjugated to an equal molar excess of fluorescent dye.

PMNs (5×10^6 cells/ml) were incubated with 3G8 or IV.3 Fab in 1 ml of 0.1 M bicarbonate buffer (pH 8.2) for 2 h on ice. After addition of GaM (30 ng/ml cross-linking GaM, fluorescence image sequences documenting the receptor aggregation were acquired using the OMA Vision CCD camera, a Zeiss Axiopert microscope, a Plan-Neofluar ×100, 1.3 NA objective and appropriate optical filters (Omega Optical).

To investigate the colocalization of FcγRIIA and FcγRIIB, PMNs were labeled with fluorescent-IV.3 and LC-biotin-3G8 Fab, which was then cross-linked with 15 mg/ml Texas Red streptavidin (Molecular Probes). After 7 min, the cells were centrifuged onto glass slides (Cytospin 2, Shandon Southern Instruments, Sewickley, PA), promptly fixed in 20°C methanol, air-dried, and mounted in glycerol with 5% acetic acid, and dried on glass slides. Digital images were acquired using a Zeiss Axiosvert microscope, a Plan-Neofluar ×100, 1.3 NA objective and appropriate optical filters (Omega Optical).

Immunofluorescence microscopy

PMNs were labeled in suspension with rhodamine-3G8 or fluorescein-IV.3 Fab and transferred to chambers with a coverslip bottom. After addition of 30 µg/ml cross-linking GaM, fluorescence image sequences documenting the receptor aggregation were acquired using the OMA Vision CCD camera, a Zeiss Axiosvert microscope, a Plan-Neofluar ×100, 1.3 NA objective and appropriate optical filters (Omega Optical).
Frustrated phagocytosis: response of human PMNs to ligation of FcγRIIA or FcγRIIB

In PMNs, too, ligation of FcγRIIA to GaM-conjugated glass via IV.3 Fab resulted in enhanced spreading relative to unstimulated controls (Fig. 3). As in PW16 cells, this response was blocked by BAPTA and genistein (data not shown). In contrast to FcγRIIA, ligation of FcγRIIB to the GaM-derivatized surface by 3G8 Fab did not elicit any morphological change in PMNs (Fig. 3). This is the only instance in which we found a discrepancy between the two FcγRs in a cellular response to a stimulus or an inhibitor. Its significance will be discussed later in the context of our model of FcγRIIB signal transduction.

Wortmannin blocks the FcγRIIA-mediated spreading response in phagocytes

Incubation of PW16 macrophages or PMNs with 10 nM wortmannin inhibited cell spreading upon FcγRIIA ligation to the derivatized glass, indicating that this response requires PI3K activity. The dependence of the spreading response on the concentration of inhibitor was quantified by image analysis (Fig. 4). We found that the wortmannin IC50 is ~2 nM in PMNs and ~23 nM in PW16 cells. The value for PMNs is in excellent agreement with that reported for inhibition of purified PI3K (25). The higher IC50 measured in macrophages may reflect an enhanced capacity of these cells to sequester or excrete wortmannin or a lower sensitivity of the murine PI3K to the inhibitor.

Cross-linking of FcγR triggers intracellular Ca2+ release

The FcγRIIA-mediated phagocytosis of opsonized erythrocytes (26) and the spreading response triggered by frustrated phagocytosis are blocked in cells loaded with the Ca2+ chelator BAPTA. To determine whether wortmannin inhibits the phagocytic response by interfering with the intracellular Ca2+ mobilization, we measured [Ca2+]i transients and the phagocytic response. Incubation of PW16 macrophages or PMNs with 10 nM wortmannin inhibited cell spreading upon FcγRIIA ligation to the derivatized glass, indicating that this response requires PI3K activity. The dependence of the spreading response on the concentration of inhibitor was quantified by image analysis (Fig. 4). We found that the wortmannin IC50 is ~2 nM in PMNs and ~23 nM in PW16 cells. The value for PMNs is in excellent agreement with that reported for inhibition of purified PI3K (25). The higher IC50 measured in macrophages may reflect an enhanced capacity of these cells to sequester or excrete wortmannin or a lower sensitivity of the murine PI3K to the inhibitor.

Frustrated phagocytosis: response of P388D1 cells to ligation of transfected FcγRIIA

Following reports about the T cell response upon ligation of membrane Ags to plastic surfaces (24), we set up a similar assay to study FcγR signaling. The GaM-coated glass did not induce spreading of resting PMNs or macrophages (Fig. 1A). However, upon addition of IV.3 Fab, surface ligation of FcγRIIA resulted in dramatic cell spreading (Fig. 1B). This response was maximal after 10 min and persisted for at least 4 h. Similar results were obtained when cells, plated on streptavidin-derivatized glass, were exposed to LC-biotin-IV.3 Fab (not shown).

This phagocytic response requires signaling by FcγRIIA. P388D1 cells expressing the FcγRIIA mutants Δ233 or Δ264, which lack the complete ITAM or the C-terminal YXXL motif, respectively, failed to spread under identical conditions (Figs. 1, C–F), even though wt and mutant FcγRs were expressed at similar levels. Quantitative image analysis shows that 10 min after FcγRIIA ligation >85% of PW16 cells measured >200 μm²/cell, whereas ~85% of P388D1 cells expressing the deletion mutants measured <200 μm²/cell (Fig. 2). These results confirm previous findings that neither mutant expressed in P388D1 cells mediates [Ca2+]i transients and phagocytosis of opsonized erythrocytes (20). Frustrated phagocytosis by PW16 cells was blocked by the Ca2+ chelator bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetate (BAPTA), loaded into the cells by a 30-min incubation with 100 μM of its acetoxymethyl ester derivative (Fig. 1G). PW16 cells incubated for 30 min with 10 μg/ml genistein, a protein tyrosine kinase inhibitor, also failed to spread, confirming that phagocytosis requires tyrosine phosphorylation (Fig. 1H).

Cross-linking of FcγR triggers intracellular Ca2+ release

The FcγRIIA-mediated phagocytosis of opsonized erythrocytes (26) and the spreading response triggered by frustrated phagocytosis are blocked in cells loaded with the Ca2+ chelator BAPTA. To determine whether wortmannin inhibits the phagocytic response by interfering with the intracellular Ca2+ mobilization, we measured [Ca2+]i transients and phagocytic response. Incubation of PW16 macrophages or PMNs with 10 nM wortmannin inhibited cell spreading upon FcγRIIA ligation to the derivatized glass, indicating that this response requires PI3K activity. The dependence of the spreading response on the concentration of inhibitor was quantified by image analysis (Fig. 4). We found that the wortmannin IC50 is ~2 nM in PMNs and ~23 nM in PW16 cells. The value for PMNs is in excellent agreement with that reported for inhibition of purified PI3K (25). The higher IC50 measured in macrophages may reflect an enhanced capacity of these cells to sequester or excrete wortmannin or a lower sensitivity of the murine PI3K to the inhibitor.
Endocytosis of cross-linked FcγRs is unaffected by wortmannin

Although the internalization of FcγRIIA bound to immune complexes is well documented, the fate of similarly cross-linked FcγRIIB has not been characterized. Based on the amounts of 125I-conjugated RaG bound to cross-linking GaM left on the surface of PMNs, we conclude that both Fab-labeled FcγRs are sequestered from the cell surface within 3 min after cross-linking by GaM (30 μg/ml) at 37°C (Fig. 6). Internalization of both FcγRs was inhibited only slightly or not at all by wortmannin concentrations as high as 100 nM, which completely blocked Ca2+ release and frustrated phagocytosis. Similarly, in PW16 cells incubated with 1 μM wortmannin there was no significant decrease in the rapid (<2 min) endocytosis of cross-linked IV.3 Fab-labeled FcγRIIIA (data not shown).

N,N-Dimethylsphingosine blocks FcγR-mediated Ca2+ release

To determine whether SK and its product S1P participate in FcγR signaling, we tested the effect of the SK inhibitor DL-threo-dihydrosphingosine, which blocked the FceRI-induced Ca2+ flux in rat mast cells (15), on the Ca2+ response of PMNs to FcγR cross-linking. However, since we found this reagent to be poorly soluble and difficult to administer to live cells, we opted to use DMS, a more soluble and potent inhibitor of SK (28, 29). Incubation of PMNs with DMS suppressed the [Ca2+]i transients triggered by specific Ab cross-linking of each FcγR, without significantly affecting the response to fMLP (Fig. 7, A and B). From a rough dose dependence of this effect, we derived an IC50 of about 0.5 nmol DMS/10⁶ cells (Fig. 7C). We use these units because the level of inhibition depended on the concentration of DMS and inversely on the density of PMNs. We believe that membrane partitioning of DMS results in its effective concentration being inversely proportional to the total membrane area in the cell suspension. For comparison to published values, because the cell density was usually about 10⁶ cells/ml, our IC50 corresponds to 0.5 μM DMS.

The PMN FcγRs may use an S1P pathway for Ca2+ release similar to that found in U937 human monocytic cells (30). In this system, activation of SK by aggregated FcγRI required the generation of phosphatidic acid by phosphatidylcholine-specific phospholipase D (PC-PLD) and was blocked by butan-1-ol. The PC-PLD catalyzes the addition of primary, but not secondary, alcohols.

FIGURE 2. Histograms of the projected areas of P388D1 cells expressing wt or truncated FcγRIIA during frustrated phagocytosis. Transfected P388D1 cells were stimulated for 0, 10, and 60 min. Analysis was performed by image-based cytometry as described in Materials and Methods. Ligated wt FcγRIIA evoked a rapid and sustained spreading response. No response was observed upon ligation of truncated FcγRIIA mutants, Δ233 and Δ264.
to the nascent phosphatidic acid by a transphosphatidylation reaction whose products, e.g., phosphatidylbutanol, do not activate SK.

To test whether PC-PLD participates in PMN FcγR signaling, we measured \([\text{Ca}^{2+}]_i\) in cells incubated with butan-1-ol or the inactive butan-2-ol (0.3%, v/v; 20 min). Butan-1-ol reduced the peak \([\text{Ca}^{2+}]_i\) following FcγRIIA cross-linking to about 30% of untreated PMNs, while leaving the response to fMLP completely unaffected (data not shown). Surprisingly, butan-2-ol induced a similar dose-dependent inhibition. Ethanol (\(1\%, \text{v/v}\)) also suppressed the FcγRIIA-induced \([\text{Ca}^{2+}]_i\) release in PMNs. In contrast, butan-1-ol had no effect on the \([\text{Ca}^{2+}]_i\) response to FcγRIIA cross-linking in U937 cells (30).

**Time course of FcγR capping**

The surface distribution of FcγRs was examined on live PMNs using fluorescent Fab. FcγRIIIB, labeled with fluorescein-3G8 Fab, appeared uniformly distributed on the surface. Upon addition of GaM, however, the receptors aggregated immediately into patches, which gradually grew in size and often coalesced into caps within 7–10 min at room temperature (Figs. 8, a–d). FcγRIIA displayed a similar behavior (data not shown). Although capping results from events occurring downstream of FcγR activation and \([\text{Ca}^{2+}]_i\) mobilization, the kinetics of the initial clustering is consistent with this process initiating the signaling cascade in PMNs.
Wortmannin disrupts FcγR capping

In addition to blocking the [Ca²⁺]₁ transients, wortmannin profoundly disrupts the FcγR aggregation in PMNs. Confocal microscope images of PMNs labeled with rhodamine-3G8 or fluorescein-IV.3 Fab, fixed 7 min after cross-linking with GaM, are shown in Fig. 9, a and b. In PMNs incubated with 30 nM wortmannin, rather than coalescing into large patches, the clusters of FcγRIIA and FcγRIIIB remain dispersed over the entire cell (Fig. 9, c and d). Thus, the PI3K activity is necessary for the large scale redistribution of cross-linked FcγRs.

Colocalization of FcγRIIA with cross-linked FcγRIIIB

The results presented thus far indicate early convergence of the signaling pathways used by the two FcγR isoforms in PMNs. We propose that FcγRIIIB clustering triggers FcγRIIA activation. However, this process requires physical interaction between FcγRIIIB and FcγRIIA. Indeed, in doubly labeled PMNs, we find that FcγRIIA comigrates with FcγRIIIB when the latter is cross-linked. Because both 3G8 and IV.3 are murine mAbs, GaM could not serve as selective cross-linker. Instead, we labeled PMNs with LC-biotin-3G8 Fab and fluorescein-IV.3 Fab and specifically cross-linked the LC-biotin-Fab with Texas Red-streptavidin. Fluorescence photomicrographs (Figs. 10, a–c) and confocal images (Figs. 10, d–e) demonstrate extensive colocalization of Texas Red (FcγRIIIB) and fluorescein (FcγRIIA). The two photomicrographs were digitized and superimposed using Photoshop (Adobe Systems, San Jose CA; Fig. 8).
with 30 mg position of cross-linked Fc linked Fc between the two Fc capping. Second, the Ca\textsuperscript{2+} or fluorescein-IV.3 (b) intracellular signal that leads to a robust Ca\textsuperscript{2+} response by spreading when Fc g elicits a response (24). We believe that this observation illustrates a key difference in the physical requirements for signal transduction by the GPI-linked Fc RIIIB and the transmembrane Fc RIIIA. The major findings of this study result corroborates a report that while T cells were stimulated by ligation of the TCR/CD3 complex to anti-CD3 IgG-coated plastic, similar ligation of the GPI-anchored proteins TAP and Thy-1 did not elicit a response (24). We believe that this observation illustrates a key difference in the physical requirements for signal transduction by the GPI-linked Fc RIIIB and the transmembrane Fc RIIIA. Phosphorylation of the Fc RIIIA ITAM is the critical event, and, indeed, Fc RIIIA is phosphorylated upon Fc RIIIB signaling by Ab-cross-linked Fc RIIIB.

Discussion
Given their similar binding properties, it is likely that both FcγR isoforms on PMNs interact with immune complexes under physiological conditions. However, to isolate the signaling function of each receptor, we have used isoform-specific mAbs to engage selectively FcγRIIA or FcγRIIB. The major findings of this study are as follows. First, without binding of FcγRIIA to ligand or cytokine priming of PMNs, cross-linked FcγRIIB delivers an intracellular signal that leads to a robust Ca\textsuperscript{2+} response and receptor capping. Second, the [Ca\textsuperscript{2+}], transients triggered by cross-linked FcγRIIB are inhibited by wortmannin and DMS with identical efficacies as those stimulated by FcγRIIA. Third, the physical disposition of cross-linked FcγRIIB, determined by immunofluorescence microscopy and internalization assay, closely parallels that of cross-linked FcγRIIA under all conditions tested. Fourth, cross-linking of FcγRIIB induces the redistribution and colocalization of FcγRIIA with aggregated FcγRIIB, as measured at the resolution afforded by optical microscopy. The only divergence between the two FcγR phenotypes was the failure of PMNs to respond by spreading when FcγRIIB was ligated to substrate-immobilized Ab.

106). Confocal images were also acquired under conditions of heterotypic cross-linking, in which both FcγRs were engaged by adding GaM (30 μg/ml) to PMNs labeled with rhodamine-3G8 Fab and fluorescein-IV.3 Fab. As expected, the distributions of the two FcγRs are perfectly correlated in these images (Figs. 10, f–g).

To test whether FcγRIIB molecules colocalize with aggregated FcγRIIA, we labeled FcγRIIB with fluorescein-3G8 and cross-linked FcγRIIA with LC-biotin-IV.3 Fab and Texas Red-streptavidin. However, under these conditions the extent of colocalization was much diminished (data not shown), probably due to the large excess of GPI-anchored receptors over their transmembrane homologues.

FIGURE 9. Wortmannin disrupts capping of cross-linked FcγRs. Confocal fluorescence images of PMNs labeled with rhodamine-3G8 (a and c) or fluorescein-IV.3 (b and d) Fab, and fixed ~7 min after cross-linking with 30 μg/ml GaM. FcγR clusters remain dispersed in PMNs preincubated with 100 nM wortmannin (c and d), but form caps in its absence (a and b).

Based on these findings, we propose that FcγRIIB signaling is mediated by FcγRIIA, which copartitions into membrane domains induced by cross-linked FcγRIIB and is thus brought into clusters for ITAM phosphorylation and activation.

FIGURE 10. The FcγRIIA colocalizes with cross-linked FcγRIIB.

a—c, Fluorescence photomicrographs of PMNs labeled with both fluorescein-IV.3 and LC-biotin-3G8 Fab after addition of Texas Red-streptavidin (15 μg/ml) to cross-link FcγRIIB. Cells were cytopsawn onto coverslips and fixed about 7 min after cross-linking, as described in Materials and Methods. a, Texas Red image; c, fluorescein image; b, merged image obtained by superposition of scanned images a and c. Confocal images of similarly prepared cells: d, Texas Red; and e, fluorescein channel. Confocal images of PMNs labeled with rhodamine-3G8 and fluorescein-IV.3 Fab after heterotypic cross-linking by GaM (30 μg/ml): f, rhodamine; and g, fluorescein channel.

Signaling by Ab-cross-linked FcγRIIB
Our results, obtained by GaM cross-linking of 3G8 Fab-bound FcγRIIB, confirm that GPI-anchored proteins are capable of intracellular signaling, as shown in T cells, and that FcγRIIB aggregation can trigger [Ca\textsuperscript{2+}], transients in PMNs (31). Ca\textsuperscript{2+} signaling by FcγRIIB was reported to require priming of PMNs with TNF or GM-CSF (10). However, we find no such need for fully functional GPI-anchored receptors.

The dependence of signaling on large scale aggregation appears to be more critical for FcγRIIB than for FcγRIIA. Again, we stress that the failure of 3G8 Fab to stimulate frustrated phagocytosis in PMNs was the only instance in which the signaling phenotypes of the two FcγRs differed. This seemingly anomalous result corroborates a report that while T cells were stimulated by ligation of the TCR/CD3 complex to anti-CD3 IgG-coated plastic, similar ligation of the GPI-anchored proteins TAP and Thy-1 did not elicit a response (24). We believe that this observation illustrates a key difference in the physical requirements for signal transduction by the GPI-linked FcγRIIB and the transmembrane FcγRIIA. Phosphorylation of the FcγRIIA ITAM is the critical event, and, indeed, FcγRIIA is phosphorylated upon FcγRIIB cross-linking (8). Formation of small clusters of...
FcγRIIA upon cross-linking with IV.3 mAb is sufficient to generate a full PMN response. However, initiation of FcγRIIA ITAM phosphorylation by FcγRIIB cross-linking requires the formation of protein/lipid domains large enough to recruit and bring into proximity an adequate number of FcγRIIA molecules. This requirement accounts for both the lag of the Ca^{2+} response to FcγRIIB cross-linking (Fig. 5A) and the inability of individually tethered and immobilized GPI-anchored proteins to trigger frustrated phagocytosis. It also explains why bivalent ligands and low valency immune complexes are ineffective at activating PMNs via FcγRIIB (10, 32).

Role of PI3K in FcγR signaling

The participation of PI3K in FcγR signaling is well documented (33) and was indeed used to show the efficacy of the inhibitor wortmannin (4). We have used wortmannin to demonstrate the congruence of signaling by the two FcγRs. The inhibition of FcγRIIB-mediated Ca^{2+} release by wortmannin implicates PI3K as a critical element for GPI-linked receptor signaling. However, because this cytosolic enzyme and FcγRIIB cannot interact directly, their coupling requires a transmembrane molecule. We believe that FcγRIIA is likely to fulfill this function, not only because it colocalizes with clustered FcγRIIB, but also because wortmannin blocks signaling by both FcγRs with identical efficacy. The molecular mechanism for recruitment of PI3K to the FcγR signaling cascade is not clear. The SH2 domains of p85, the regulatory subunit of PI3K, have been shown to recognize tyrosine-phosphorylated YXXM sequences (34, 35). Because FcγRIIA lacks this motif, it may interact with PI3K via an adapter molecule. In platelets, the tyrosine kinase Syk was shown to associate with phosphorylated FcγRIIA and was proposed to recruit PI3K to the activated receptors (36). However, direct interaction between p85 and FcγRIIA cannot be ruled out, because p85 was found to bind to doubly phosphorylated ITAMs of the ζ- and ε-chains of CD3 (37, 38). The PI3K from PMNs was also found to associate in vitro with a fusion protein consisting of GST and the cytosolic domain of FcγRIIA (39).

In agreement with our observation that wortmannin is unable to block the endocytosis of cross-linked FcγRs, PI3K inhibition does not block the internalization of PDGF receptors, but it interferes with their trafficking to lysosomal compartments (40). These findings suggest that the lipid products of PI3K may play a critical role in FcγR signaling. Polyphosphorylated and, in particular, 3-hydroxyphosphorylated inositol lipids participate in regulating endocytic transport and membrane trafficking (41). Phosphatidylinositol 3,4,5-trisphosphate (P(3,4,5)P3) activates phospholipase C-γ1 by binding to its pleckstrin homology or its SH2 domain (42, 43). The P(3,4,5)P3, also functions in the activation of c-akt by binding to its pleckstrin homology domain, which is required for phosphorylation of c-akt, and by directly stimulating a specific kinase (44). Whether PI3K interacts with phosphorylated FcγRIIA directly or via an intermediate, our results indicate that it has an identical functional role in both FcγRIIA and FcγRIIB signaling.

Inhibition of [Ca^{2+}], transients by DMS

Following a report of a much weaker IP3 release after FcγR cross-linking than after fMLP stimulation (11), later investigations have yielded conflicting results (10, 12, 13). The amount of IP3 was recently confirmed to be much smaller in PMNs activated by FcγRIIA aggregation than by fMLP stimulation and almost negligible after FcγRIIB cross-linking (14). Meanwhile, S1P has been found to mediate intracellular Ca^{2+} release in 3T3 fibroblasts (45) and the autocrine stimulation of platelets (28). In permeabilized fibroblasts, the Ca^{2+} response to S1P was not blocked by heparin, an IP3 antagonist (46). In rat mast cells, inhibition of SK abolished the Ca^{2+} release following FceRI stimulation while leaving the IP3 pathway intact (15). However, in U937 cells, FcγRI mobilized Ca^{2+} by activating PC-PLD and SK, whereas FcγRIIA triggered a substantial IP3 production (30).

We have shown that DMS, a competitive inhibitor of SK, blocks Ca^{2+} release in PMNs stimulated by FcγR cross-linking, but not by fMLP. In contrast to our results, DMS was recently reported to inhibit the PMN Ca^{2+} response to fMLP with an IC50 of ~5 μM (47). However we found the FcγR response to be 10-fold more sensitive to inhibition, and interpreted any effect on the fMLP-triggered Ca^{2+} release at DMS concentrations >2 μM as nonspecific toxicity. The SK activation pathway in PMNs remains undefined, because the inhibition studies with the butanol isomers failed to confirm the participation of PC-PLD demonstrated in the case of FcγRI (30). It also seems likely that FcγRIIA uses distinct signaling pathways in different cell types, because IP3 release is substantial in U937 cells (30) but only minimal in PMNs (14). However, as for wortmannin, the similar efficacy with which DMS inhibits PMN activation by both FcγRs indicates that they share a common signaling pathway.

Because S1P and IP3 mobilize Ca^{2+} from the same thapsigargin-sensitive stores (11, 46), differences in Ca^{2+}-dependent PMN activation via FcγR and fMLP receptors may arise from a different cellular compartmentalization of their respective second messengers. As opposed to the water-soluble IP3 molecule, S1P is probably mostly membrane bound. Thus, SK activation by FcγRs may generate a more localized response, suitable for mediating phagocytosis, than that produced by IP3 release, which may mediate whole cell responses, such as chemotaxis.

Functional dependence of FcγRIIB on signaling by FcγRIIA

Because the pattern of FcγRIIA aggregation induced by FcγRIIB cross-linking is identical with that induced by direct FcγRIIA cross-linking, we propose that a major component of the signal generated by FcγRIIB is transduced by FcγRIIA. In our model, FcγRIIB relies on the ITAM of FcγRIIa for signal transduction and, thus, disruption of this motif should impair the signaling capacity of both FcγR isotypes. A suitable biological system to replicate the PMN FcγR signaling machinery is not at hand. Human PMNs are short lived and not amenable to conventional molecular biological approaches, whereas FcγRIIB transfected into heterologous systems is often expressed in a nonfunctional or transmembrane form. Nevertheless, Green et al. expressed both FcγRs in Jurkat T cells and showed that, as in PMNs (9), co-cross-linking of the two receptors elicited a synergistic enhancement of the [Ca^{2+}], transient relative to that triggered by cross-linking of FcγRIIA alone (48). This effect required expression of the ITAM of FcγRIIA and of the GPI anchor of FcγRIIB. However, it is unclear whether this cell line is a reliable model of human PMNs, because the [Ca^{2+}], transients triggered by cross-linking FcγRIIB or the more abundant endogenous CD59 were weak, slow rising, and completely abolished by chelation of extracellular Ca^{2+}. Moreover, the TCR complex expressed in this cell line may contribute to signaling by the exogenous FcγRIIB. In T cells, the ITAM-bearing TCR mediates signaling by cross-linked GPI-anchored proteins (16, 49). Further work in this area is required.

Physical models of FcγRIIB function

In formulating a model for FcγRIIB signaling we note that in PMNs, as in T cells, Ca^{2+} is released upon cross-linking of various GPI-anchored proteins (50). The signaling capacity of these diverse proteins may derive from their common structural element,
the GPI anchor, rather than from specific protein-protein interactions. Focusing on the glycan portion of GPI, Petty and colleagues found that FcγRIIIB-triggered \([\text{Ca}^{2+}]_i\) transients and superoxide production were induced by high concentrations of \(N\)-mannose or \(N\)-acetyl-\(\beta\)-glucosamine, each part of the conserved core structure of GPI anchors (51). Moreover, \(N\)-acetyl-\(\beta\)-glucosamine disrupted cocapping of FcγRIIIB with CD11b/CD18, a \(\beta_2\) integrin also known as complement receptor 3 (18). Because complement receptor 3 contains a lectin-like site that could recognize GPI, they proposed that FcγRIIIB signaling results from binding to the \(\beta_2\) integrin (52). Indeed, integrins are known to modulate FcγR-mediated PMN activity (53). FcγRIIIB may also interact specifically with the formyl peptide receptor, because soluble immune complexes or 3G8 Fab block fMLP-induced chemotaxis, but not the response of PMNs to other chemotactic stimuli (54). However, based on its physical proximity, signaling capacity, and the evidence of functional interactions between GPI-anchored proteins and ITAM-bearing immunoreceptors, we propose that FcγRIIA is the primary signal transducer for FcγRIIIB in PMNs.

Aggregated FcγRIIIB may recruit FcγRIIA via interactions within the membrane hydrophobic core, GPI-anchored proteins are selectively enriched, together with sialoglycolipids, glycolipids, and cholesterol, in detergent-insoluble membrane complexes isolated from cold Triton X-100 cell lysates (55, 56). Doubly acylated Src family tyrosine kinases are also found in these complexes (57). First identified with caveolae, these complexes have since been isolated from cells lacking these structures (58). These and other similar findings led Simons and Ikonen to propose that these lipid-protein complexes form microdomains or functional “rafts” that may participate in trafficking and sorting of membrane components, and function as integrated signaling assemblies (19). Evidence of clusters of GPI-anchored proteins, obtained by fluorescence microscopic measurements of resonance energy transfer and by chemical cross-linking (59, 60), supports the existence of rafts in vivo. However, the long term stability of these domains is not absolutely required by the model. Indeed, rafts may be transient dynamic entities stabilized by cooperative interactions formed upon aggregation of GPI-anchored proteins (61). Thus, random dispersion of FcγRIIIB on the surface of resting PMNs is compatible with raft formation after cross-linking.

Two fundamental questions remain outstanding. The first concerns the identity of the molecular interactions leading to formation of rafts and inclusion of lipid-linked proteins. The second, relevant to our model of FcγRIIIB signaling, concerns the mechanism for recruitment of transmembrane proteins, such as FcγRIIA, to the rafts.

With regard to the first issue, the composition and physical properties of the plasma membrane are critical for microdomain stability. Depletion of cellular cholesterol destabilizes the detergent-insoluble complexes and inhibits signal transduction by GPI-anchored proteins in T cells (62). Incubation of T cells with polyunsaturated fatty acids causes inhibition of the \(\text{Ca}^{2+}\) response to stimulation via CD3 and CD59 and displacement of the Src family tyrosine kinase Lck from the detergent-insoluble complexes (63). Mixtures of cholesterol and sphingomyelin or dipalmitoylphosphatidylcholine form membranes that enhance the detergent insolubility of GPI-anchored proteins (64). The current hypothesis for the physical basis of these phenomena invokes the formation of cholesterol and sphingolipid-rich domains where the bilayer exists in the liquid-ordered \((L_o)\) phase (65, 66). In this phase, the lipid chains are highly ordered, as in the gel phase, yet the lipids remain free to diffuse. These unusual properties are thought to facilitate partitioning of proteins with suitable lipid anchors. This idea was tested in vitro using placental alkaline phosphatase (67), whose GPI anchor contains saturated palmitic and stearic acyl chains (68). However, the GPI-anchored acetylcholinesterase and CD59 from human RBCs are also detergent insoluble (69, 70) despite containing highly unsaturated 2-acyl chains (71, 72). Thus, unsaturated lipid anchors appear to be compatible with incorporation into rafts. Although the structure of the GPI anchor of FcγRIIIB is unknown, it is unlikely to exhibit drastically different properties. Indeed, preliminary experiments indicate that FcγRIIIB, but not FcγRIIA, in resting PMNs segregates into detergent-insoluble membrane fractions (our unpublished observations).

Regarding the second issue, we admit that evidence for inclusion of transmembrane proteins in rafts is still weak. However, the following observations indicate that single-pass transmembrane proteins may indeed associate with rafts. Influenza virus hemagglutinin (HA) and CD44, both type I proteins like FcγRIIA, and influenza virus neuraminidase, a type II protein, partition in detergent-insoluble complexes (73–75). Glycoporphin, a type I glycoprotein abundant in RBCs, promotes formation of cholesterol-rich domains in model membranes (76).

Two properties of transmembrane domains have been suggested to determine the affinity of membrane proteins for rafts: their length and the presence of specific amino acids. The first property was invoked in the mechanism of protein sorting within the Golgi apparatus. The transmembrane domains of type II proteins retained in this organelle were found to be shorter (15 aa), on the average, than those of analogous proteins delivered to the plasma membrane (20 aa). This effect was attributed to the thicker hydrophobic core of trans-Golgi and plasma membranes, a result of their enrichment in cholesterol and saturated sialoglycolipids (77). For thermodynamic reasons, proteins tend to partition where the hydrophobic thickness of the bilayer matches the length of their hydrophobic transmembrane domain (78). This mechanism of protein sorting has received experimental and theoretical support (79, 80).

A role for the transmembrane domain sequence in determining the affinity of proteins for rafts has been inferred from mutagenesis studies on HA and neuraminidase (73, 75). Although no targeting motif was identified, the work on HA unveiled a requirement for isoleucine and leucine residues to be in contact with the outer membrane leaflet. A preference for large hydrophobic residues in the N-terminal half of the transmembrane helix of type I proteins had been previously noted (81).

Although these requirements are rather loose, the FcγRIIA transmembrane domain satisfies both of them. It is predicted to be 24 aa long, exceeding the value cited by Bretscher and Munro, and its N-terminal sequence is rich in \(\beta\)-branched hydrophobic residues (I\(^{199}\)VAVVI). Moreover, a cysteine, Cys\(^{241}\), resides at the cytoplasmic membrane-water interface predicted by the stop transfer sequence of basic residues, R\(^{242}\)KKR (82). Cysteines at this location are targets for palmitoylation (83). Although it is unknown whether such modification occurs in vivo, acylation could increase the affinity of FcγRIIA for rafts.

Although further investigation is necessary to validate our model, our data provide new insight into the signaling mechanism of FcγRIIIB and the functional architecture of the PMN plasma membrane. Although activation of many receptors relies on intramolecular conformational changes or oligomerization, the signaling capacity of GPI-anchored proteins may derive from their ability to induce the formation of microdomains of defined composition within the plasma membrane. Thus, aggregation of FcγRIIIB may lead to formation of supramolecular assemblies comprising all the components, including FcγRIIA, necessary for generating cellular responses.
Acknowledgments

We thank Heikki Väänänen for expert assistance with microscopy and Josef Eisinger for valuable discussions.

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