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CONVERGENT Fc-GAMMA RECEPTOR SIGNALING IN THE HUMAN NEUTROPHIL: EVIDENCE FOR ROLE OF GPI-ANCHORED PROTEINS

by

FRANK Y. S. CHUANG

A dissertation submitted to the Graduate Faculty in Biomedical Sciences, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

CONVERGENT Fcγ RECEPTOR SIGNALING IN THE HUMAN NEUTROPHIL: EVIDENCE FOR ROLE OF GPI-ANCHORED PROTEINS

by

Frank Y. S. Chuang

-Preceptors-

Massimo Sassaroli, D.Sc.*, Jay Unkeless, Ph.D.†, and Josef Eisinger, Ph.D.*

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The recognition of immune complexes and opsonized bacteria by polymorphonuclear leukocytes (PMNs or neutrophils) is mediated by receptors which bind to the constant (Fc) portion of immunoglobulin (IgG). Within the Fcγ receptor family, the FcγRIIIB isoform is unique for its attachment to the cell membrane via glycosyl-phosphatidylinositol (GPI). The molecular basis for transmembrane signaling by FcγRIIIB (as well as by other GPI-anchored receptors) has been unclear. Spectrofluorimetric data obtained using the calcium indicator Indo-1 demonstrate that direct antibody crosslinking of FcγRIIIB on isolated human PMNs triggers a rapid, transient rise in intracellular calcium concentration, comparable to that exhibited by crosslinking the membrane-spanning FcγRIIA isoform. Inhibition by wortmannin and by N,N-dimethylsphingosine of signaling through either FcγRIIA or FcγRIIIB shows similar dose response, suggesting that both receptors converge on a phosphatidylinositol 3-kinase and sphingosine kinase-dependent pathway. Immunofluorescence microscopy and internalization assays demonstrate that separately crosslinked FcγRIIIB and FcγRIIA show similar patterns of movement on living cells; and

furthermore, without direct involvement in crosslinking, FcγRIIA co-localizes with aggregated FcγRIIIB. FcγRIIA is thus implicated as the essential transmembrane element required for signaling by GPI-anchored FcγRIIIB.

The physical nature of association between FcγRIIA and FcγRIIIB remains to be elucidated. The observation that dialkyl-indocarbocyanine (DiI) membrane probes also co-localize with clustered FcγRIIIB *in vivo*, suggests that the physical properties of the lipid bilayer in the region of the GPI-anchored receptor are significantly different from that of bulk membrane, and that other molecules (including FcγRIIA) may selectively partition into these regions. A physical model is proposed in which clustered GPI-anchored proteins form a lipid domain comprised of sphingolipids and cholesterol, and organized around hydrophobic interactions. The partitioning and enrichment of FcγRIIA within these domains lead to the subsequent initiation of intracellular signaling. This model provides a rationale for the intriguing observation that crosslinked, but immobilized FcγRIIIB does not stimulate neutrophil activity.

Other experiments to directly assess the functional dependence of FcγRIIIB on FcγRIIA, and to examine receptor proximity with nanometer-scale resolution, are also described.

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In memory of my former mentor,

JACOB I. FABRIKANT, MD, PHD

who guided me on the path towards
research medicine and investigative science.

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INTRODUCTION

The multigene family of receptors for the constant or Fc portion of IgG (henceforth called Fcγ receptors or FcγR) bridge classic humoral immunity with cell-dependent defense mechanisms. In polymorphonuclear leukocytes (PMNs or neutrophils), low-affinity FcγR crosslink with immune complexes or opsonized targets to trigger a pleiotropic response which includes phagocytosis, degranulation, oxidative burst and antibody-dependent cell cytotoxicity (ADCC). One of the earliest intracellular signaling events detected following FcγR stimulation is the tyrosine phosphorylation of several cytosolic intermediates. However, FcγR in themselves do not exhibit intrinsic tyrosine kinase activity. Rather, the initiation of the signaling cascade relies on a unique signaling module known as the immunoreceptor tyrosine-based activation motif (ITAM). [1] The canonical ITAM is identified by a pair of YXXL sequences separated by 6-8 amino acids, and is present in single or multiple copies in the cytoplasmic domain of multichain T cell and B cell antigen and Fc receptors. [2] The tyrosine-phosphorylation of ITAMs by *src* family kinases under conditions of FcγR crosslinking has been shown to be essential for binding and subsequent activation of downstream nonreceptor *syk* protein tyrosine kinases. [3]

As illustrated in **Figure 1**, the structural arrangements of multichain antigen and Fcγ receptors are such that the immunoglobulin-like extracellular ligand binding domain and the intracellular ITAM effectors are usually contained in separate subunit chains. Curiously, the two FcγR isoforms constitutively expressed on the surface of the human neutrophils both represent exceptions to this rule, albeit in opposite ways: FcγRIIA (CD32) consists of a single Class I glycoprotein which contains both the ligand-

binding domain and a slightly modified ITAM domain (in which the two YXXL sequences are separated by 12 amino acids), on the same transmembrane peptide. [4]

In contrast, FcγRIIIB (CD16B) consists of an immunoglobulin-like Fc binding domain attached to the outer leaflet of the plasma membrane by a C-terminal amino acid-linked glycosyl-phosphatidylinositol (GPI) anchor [5-7], and therefore conspicuously lacks both transmembrane and cytosolic portions. However, despite its consequent lack of an associated ITAM signaling module, FcγRIIIB most likely plays a predominant role in binding immune complexes, with a ten-fold higher level of expression than FcγRIIA, as well as a relatively higher binding affinity to IgG.

The co-expression of two Fcγ receptor isoforms in human neutrophils is puzzling in several ways. Since FcγRIIIB and FcγRIIA have similar ligand binding characteristics and presumably are both engaged in crosslinking to naturally occurring immune complexes, it is unclear why two receptor isoforms are expressed where only one is apparently required. Ideally the solution to this problem would be that the two FcγR species possessed inherently different signaling properties, but this remains to be demonstrated. This rationale, however, begs the question of transmembrane signaling by FcγRIIIB, which is paradoxical in itself, since GPI-anchored molecules are physically restricted from communicating directly with downstream signaling elements in the cytosol.

It follows that the primary objectives of this dissertation project have been to determine the functional relation of FcγRIIIB and FcγRIIA, and to

characterize the involvement of GPI-anchored proteins in transmembrane signaling, using the human neutrophil as a model system. This investigation makes extensive use of isotype-specific monoclonal antibodies to directly crosslink either FcγRIIIB or FcγRIIA, in order to differentiate the relative signaling phenotypes of the two receptor species. We observe a striking similarity in the pharmacologic profiles of FcγRIIIB and FcγRIIA-mediated intracellular calcium release and their susceptibility to inhibition of phosphatidylinositol 3-kinase and sphingosine kinase, and propose that FcγRIIIB and FcγRIIA converge on a common signaling pathway at a very early stage following engagement of the receptors.

To validate the functional link between FcγR, we also conducted parallel studies using immunofluorescence microscopy and internalization assays to demonstrate a probable physical association between these Fcγ receptor species as well. These results, together with findings from other investigators, led to the development of a physical model in which crosslinked FcγRIIIB triggers intracellular calcium release by forming specialized lipid domains which in turn recruit FcγRIIA (containing the ITAM effector module) as the necessary transmembrane signaling element. Although the specific properties which distinguish these proposed lipid domains from bulk membrane are unclear, the proposed mechanism provides a basis for the involvement of other signaling molecules (hence, multiple signaling pathways) in FcγR activation. Furthermore, the GPI-anchoring structure is implicated as the important signaling moiety of FcγRIIIB, thus explaining the empirical observation that other GPI-anchored proteins can also stimulate neutrophil activity. We suggest that this model

may in fact represent a new signaling paradigm for receptors which are GPI-anchored.

BACKGROUND

The first half of this section summarizes what is known about FcγR signaling (mainly by FcγRIIA via the intracellular ITAM effector), followed by separate discussions on the involvement of the calcium second messenger and a relatively novel but ubiquitous enzyme, phosphatidylinositol 3-kinase, in neutrophil activation. The second half is devoted to the issue of signaling by FcγRIIIB, in the context of previous research examining the structure of GPI-anchor proteins and possible modes of intermolecular interaction which might allude to mechanisms of signal transduction.

FcγRIIA signal transduction

Despite the structural variations which distinguish FcγRIIA from other ITAM-containing antigen and Fc receptors, the molecular basis for signaling by FcγRIIA is consistent with the mechanism characterized for other receptors which contain the ITAM effector module. In general, the event which determines the activation of immune receptors (such as FcγRIIA) is not simply the recognition and monovalent binding of receptor to ligand, but the clustering and aggregation of these receptors by crosslinking to immune complexes or polyvalent ligands. This supports the physiological role of FcγR in triggering neutrophil immune response upon contact with large immune complexes or opsonized targets, rather than to soluble IgG in the bloodstream. Indeed, FcγRIIA has been shown to require that IgG be at least dimerized, in order to bind with low avidity (K_a = 1~3 x 10^6 M^{-1}, [8])

As previously mentioned, FcγRIIA contains only one C-terminal ITAM module in its cytoplasmic tail. While the consensus ITAM sequence consists of two YXXL repeats separated by 6-8 amino acids, this intervening sequence is

extended to 12 amino acids in the signaling motif for FcγRIIA. [9] The functional importance of ITAM in FcγRIIA signaling was demonstrated by Odin and coworkers, who used both wildtype and truncated deletion mutants of human FcγRIIA transfected in murine P388D1 macrophages to demonstrate that, while the deletion of the carboxyterminal YXXL motif (Δ264) from FcγRIIA prevented phagocytosis of IgG-sensitized erythrocytes and intracellular Ca^{2+} release, the complete removal of the ITAM in Δ233-FcγRIIA blocked all remaining functionality, including the receptor's ability to stimulate intracellular tyrosine phosphorylation and endocytosis of small immune complexes. [10]

The link between tyrosine-phosphorylation of the ITAM and the progression of downstream intracellular signaling was demonstrated in T lymphocytes using synthetic non-hydrolyzable phosphotyrosyl peptide analogs of the third ITAM module in the ζ chain of the T cell antigen receptor (TCR)/CD3 complex. (ref. Figure 1, [11]) The association of zeta-chain associated protein (ZAP-70, a *syk* family kinase) with activated TCR was thus shown to be competitively inhibited by the presence of an ITAM peptidomimetic which was phosphorylated at both tyrosine residues, while exogenous ITAM peptides phosphorylated in only one of the two tyrosine positions had no observable effect. The blocking of ZAP-70 association with the distal ITAM of TCRζ coincided with the inhibition of ZAP-70 phosphorylation and activation by TCR, as well as a reduction in the extent of tyrosine phosphorylation of a number of cytosolic intermediates, suggesting that ZAP-70 was the necessary signaling element immediately downstream of activated TCR.

Evidence for the involvement of *src* family kinases in the phosphorylation of ITAM *in vivo* includes the rapid induction of *lck* and *fyn* activity following TCR stimulation [12], as well as *blk*, *lyn* and *fyn* activation triggered by the B cell antigen receptor (BCR). [13] Screening of random synthetic peptide libraries for potential *src* kinase substrates identified a preferred sequence for tyrosine phosphorylation which matched the YXXL motif of ITAM. [14] The specificity of certain *src* kinases for particular ITAM sequences may provide a basis for the regulation of multiple signaling pathways by a single receptor containing several ITAM modules. The *src* family kinase(s) which recognize and bind specifically to FcγRIIA have not been identified conclusively, but *hck*, *lyn*, and *fgr* have all been demonstrated to bind other FcR ITAMs. (reviewed in Isakov, [2])

The model for signaling by FcγRIIA and other ITAM-containing receptors resembles somewhat the mechanism of activation for receptor tyrosine kinases (such as the EGF or insulin receptors), in that receptor crosslinking (or dimerization, as the case may be) results in the arrangement of receptor-associated kinases to a suitable configuration which allows subsequent phosphorylation of the relevant tyrosine residues to occur.

Involvement of PI 3-kinase in neutrophil activation

Mounting evidence implicates phosphatidylinositol (PI) 3-kinase as a central component in many diverse signaling systems, including those involving EGF and PDGF receptor protein tyrosine kinases, non-receptor protein tyrosine kinases such as *src* and *fyn* [15-17], as well as FcγRIIIA (CD16A) in natural killer (NK) cells. [18] The enzyme was also shown to be a downstream target of *ras* [19], mediating PDGF and insulin-dependent

$pp70^{S6K}$ activation. [20] PI 3-kinase is a heterodimeric molecule, consisting of an 85 kDa regulatory subunit (p85) that contains one SH3 and two SH2 domains, but no catalytic activity [21-23], and a 110 kDa catalytic subunit (p110) which phosphorylates PI, PI 4-P, and PI 4,5-P_2 on the D-3 position of the inositol ring. [24] Different PI 3-kinase isoforms have purportedly been identified, based on experimentally-determined characteristics of the catalytic subunit, and may be involved in different signaling systems [25-27]; however, the classification of these separate enzymes is still unclear, and a standard nomenclature has not yet been established.

The discovery of the seemingly ubiquitous involvement of PI 3-kinase in such diverse signaling pathways was aided largely by the experimental application of a fungal metabolite called wortmannin, which potently and selectively blocks PI 3-kinase activity with an estimated K_i = 5 nM in guinea pig neutrophils. [28] Wortmannin is highly membrane-permeable and effectively inhibits many cellular processes, including chemotactic peptide receptor-mediated superoxide burst in neutrophils [28], insulin-induced glucose transport and antilipolysis in rat adipocytes [29], as well as FcγR-mediated phagocytosis in U937 cells and guinea pig neutrophils. [30] Wortmannin has been shown to irreversibly block PI 3-kinase activity by binding covalently to Lys^{802} of the catalytic p110 subunit, situated near the ATP-binding region of the enzyme. Saturating concentrations of PI (4,5) bisphosphate and ATP, however, were shown to compete with wortmannin binding to PI 3-kinase. [31]

The precise role of PI 3-kinase in signaling by FcγR is still unclear, although several lines of evidence support a role for p85 as an adaptor

molecule, facilitating the assembly of multimeric signaling complexes associated with activated PDGF receptor [32], insulin receptor [33], and B cell antigen receptor. [34] PI 3-kinase is not predicted to interact directly with FcγRIIA, since previous studies have shown that the SH2 domains of p85 direct the preferential binding of PI 3-kinase to proteins such as CD28 (a surface antigen required for T cell proliferation), which contain a tyrosine-phosphorylated YXXM motif. [35-37] However, the possibility of a direct interaction between p85 and phosphorylated FcγRIIA cannot be ruled out, since p85 was shown to bind to the membrane-proximal ITAM (YXXL) in the ζ chain of CD3, which augments signaling by the T cell receptor. [38] Also, *in vitro* studies using recombinant fusion proteins of glutathione-S-tranferase (GST) with the cytoplasmic domain of FcγRIIA (including the ITAM motif), demonstrated an association with neutrophil cytosolic PI 3-kinase, which depended on phosphorylation of the C-terminal Tyr^{253} of FcγRIIA. [39] The affinity of PI 3-kinase for non-YXXM motifs might be augmented if bi-dentate binding can take place, as demonstrated by mutagenesis studies which show that both Tyr^{170} and Tyr^{181} of the CD3-ε ITAM must be phosphorylated in order to bind the tandem SH2 domains of p85. [38]

The work presented in this dissertation extends the observation that PI 3-kinase is involved in human FcγR-mediated processes, by demonstrating that FcγRIIIB and FcγRIIA signaling are equally susceptible to inhibition by wortmannin. Furthermore, evidence from separate immunofluorescence microscopy studies and assays examining Fcγ receptor internalization suggest that this aspect of the cell response may also involve the activity of the p110 catalytic subunit.

Involvement of calcium in neutrophil activation

The initiation of the tyrosine phosphorylation cascade following Fc receptor activation leads to the activation of another essential intracellular second messenger system. The transient rise of free calcium concentration in the cytosol, $[Ca^{2+}]_i$, triggered by the activation of FcγR or the G protein-coupled chemotactic peptide (formyl Met-Leu-Phe or fMLP) receptor, is required for a variety of functions, including chemotaxis, production of oxidative metabolites, exocytosis and phagocytosis. [40-42] For these reasons, as well as other technical considerations which will be discussed later in the section on Experimental Design, the measurement of $[Ca^{2+}]_i$ by Indo-1 spectrofluorimetry was the method of choice to monitor FcγR-mediated neutrophil activation and to compare the relative signaling phenotypes of FcγRIIIB and FcγRIIA.

In phagocytic cells, as in myocytes, low resting $[Ca^{2+}]_i$ levels (< 50nM) are maintained by the constitutive activity of calcium pumps functionally related to the Ca^{2+}-ATPase in muscle sarcoplasmic reticulum, which transport calcium ions outside the cell, across the plasma membrane, or sequester it in specialized intracellular storage compartments. Unlike the sarcoplasmic reticulum, however, the calcium storage compartment in the neutrophil is insensitive to caffeine, thus excluding ryanodine-sensitive channels from consideration as possible regulators of calcium release. [43] Rather, stimulation of the fMLP receptor in neutrophils has been shown to lead to mobilization of intracellular calcium via the conventional inositol (1,4,5)-trisphosphate (IP_3)-dependent pathway, in which soluble IP_3 released from the hydrolysis of PIP_2 by activated phospholipase C beta (PLC-β) binds to IP_3-gated calcium release channels in cytoplasmic storage vesicles. [44]

Under conditions where thapsigargin inhibits re-uptake of cytosolic calcium by blocking Ca^{2+}-ATPase activity, the transient rise in $[Ca^{2+}]_i$ triggered by crosslinking FcγR can be attenuated by prior stimulation of the fMLP receptor (and vice versa), suggesting that the calcium messenger mediating both events is released from the same intracellular storage compartment. Since these compartments (which were named "calciosomes" by Lew and colleagues [45]), were already found to contain IP3-sensitive calcium channels, the assumption has been that FcγR-stimulated elevations in $[Ca^{2+}]_i$ must also originate by an IP3-dependent mechanism. However, several lines of evidence suggest that a different regulatory pathway exists for FcγR. In particular, whereas both IP3 production and the subsequent elevation in $[Ca^{2+}]_i$ triggered by fMLP are blocked by pertussis toxin (PTX, which is indicative of signaling coupled to G protein activity), the transient rise in $[Ca^{2+}]_i$ evoked by FcγR crosslinking is PTX-insensitive. While this finding alone did not rule out the possibility that FcγR-triggered calcium release is mediated by PLC-γ (whose activity is also known to release IP3), Rosales and Brown also demonstrated that IP3 production following FcγR stimulation was disproportionately small, compared to the amount released by activation of the fMLP receptor, and was inhibited by pertussis toxin. [44]

I present preliminary evidence which supports an alternative mechanism for FcγR-mediated intracellular calcium release, involving a novel second messenger, sphingosine 1-phosphate. This presumably membrane-bound molecule was demonstrated to activate Ca^{2+}-dependent events in platelets [46], and has been shown to mobilize intracellular Ca^{2+} in rat mast-cells triggered by the FcεRI antigen receptor, which proceeds via an

IP3-independent mechanism. [47] Using a competitive inhibitor of sphingosine kinase, N,N-dimethylsphingosine, to block sphingosine 1-phosphate production, I demonstrate the selective inhibition of both FcγRIIIB and FcγRIIA-mediated $[Ca^{2+}]_i$ transients, but not those triggered by fMLP.

Functional relation of FcγRIIIB and FcγRIIA

In light of the relatively straightforward transmembrane signaling mechanism of FcγRIIA, and the extent to which crosslinking of FcγRIIA alone can trigger neutrophil response, it is unclear how the GPI-anchored receptor isoform further contributes to the activation of PMNs by immune complexes. Compared to FcγRIIA, the GPI-linked FcγRIIIB has a ten-fold higher level of expression (135,000 vs. 10,000 receptors/cell, [48, 49]) and binds IgG with relatively higher affinity ($K_a \approx 4 \times 10^6\ M^{-1}$, [50]), leading one to speculate that perhaps FcγRIIIB serves only to improve ligand binding efficiency for signaling via FcγRIIA, rather than function as a *bona fide* receptor, capable of initiating a cellular response.

Arguing against this polar view, numerous studies have demonstrated that crosslinking FcγRIIIB, in the absence of direct ligation of FcγRIIA, does in fact lead to neutrophil activation. However, the relative contribution of the two receptor species in stimulating an immune response has been subject to debate. Based on experiments using autoimmune anti-FcγR IgM to trigger human neutrophils, FcγRIIIB was shown to mediate intracellular calcium flux and degranulation, since the specific IgM which was used did not bind strongly to targets expressing only FcγRIIA. [51] Functional studies on neutrophils from patients with paroxysmal nocturnal hemoglobinuria (which arises from defects in GPI-anchor biosynthesis and results in much

lowered expression of FcγRIIIB), show normal superoxide burst in response to IgG complexes [52], while other investigations demonstrate that direct antibody crosslinking of FcγRIIIB also induces respiratory burst in normal neutrophils. [53] Separate studies using either anti-FcγR mAb-coated erythrocytes or B cell hybridomas expressing anti-FcγR surface IgG [54] to trigger neutrophil activity, showed that both cytolytic activity (ADCC) and phagocytosis are more effectively mediated by FcγRIIA than by FcγRIIIB. [55, 56]

Although these investigations successfully demonstrate that FcγRIIIB is capable of signaling, the functional role that can be attributed to the GPI-anchored receptor on the basis of these observations is, at best, redundant next to that of FcγRIIA. Other observations suggest instead that FcγR signaling involves multiple signaling pathways. The induction of actin polymerization by FcγRIIA crosslinking, for example, was shown to be mainly calcium independent, whereas Ca^{2+} chelation by BAPTA abrogated this response in FcγRIIIB-induced cells. [57] Moreover, the stimulation of oxidative burst by crosslinking FcγRIIIB is refractory to inhibition by pertusss toxin, while the same activity triggered by crosslinked FcγRIIA is pertussis toxin-sensitive. [58]

Still other investigations support a synergistic relation between the two receptor species. After demonstrating that heterotypic FcγR crosslinking was more effective than crosslinking either FcγR isoform alone at stimulating neutrophil phagocytosis of IgG-coated erythrocytes, Kimberly and coworkers showed that oxidative metabolites secreted by the neutrophil upon FcγRIIIB stimulation exerted a positive autocrine/paracrine effect on FcγRIIA mediated

phagocytosis. [59] The observation that FcγRIIA signaling can be potentiated by FcγRIIIB crosslinking suggests a component of signaling by the GPI-anchored receptor which is separate from FcγRIIA. Although the physical model which we propose for FcγRIIIB signaling does not preclude the possible involvement of alternate signaling mechanisms, a major conclusion of this present study is that the bulk of signaling by FcγRIIIB falls on a pathway which converges with that of FcγRIIA.

Evidence for signaling by GPI-anchored proteins

Underlying the question of signaling by FcγRIIIB is a much larger issue of the still-undetermined role that GPI-anchored molecules play in plasma membrane physiology. Evidence for the covalent attachment of a membrane protein to a glycolipid moiety was first obtained more than 40 years ago, through studies of the pathogenesis and etiology of anthrax, which showed that a peculiar symptom of livestock infected with *Bacillus anthracis* was a pronounced elevation of alkaline phosphatase in the bloodstream. [60] The soluble factor secreted by *B. anthracis* which caused release of alkaline phosphatase from tissues was determined to be a novel phospholipase C, specific for phosphatidylinositol (hence, PI-PLC). [61] After other cell surface enzymes, such as 5'-nucleotidase [62] and acetylcholinesterase [63], were also found to be released from intact cells by PI-PLC hydrolysis, the identification of *myo*-inositol near the C-terminal anchoring region of purified acetylcholinesterase [64] and alkaline phosphatase [65] confirmed the identity of a new class of membrane proteins which were covalently linked to phosphatidylinositol. The method of cleaving proteins by exogenous PI-PLC (or also by phospholipase D) has since been used to identify a diverse group of functionally unrelated membrane proteins as GPI-anchored, including decay

accelerating factor (DAF, [66]), Thy-1 [67], carcinoembryonic antigen (CEA, [68]), the scrapie prion protein [69], the folate receptor [70] and urokinase-type plasminogen activator receptor (UPAR) [71, 72], as well as FcγRIIIB. [5-7]

The biosynthesis of the GPI anchor in mammalian cells, as well as the signal peptide sequences which identify nascent polypeptides for translocation and covalent attachment to preformed anchors present on the luminal surface of the endoplasmic reticulum, has been fairly well characterized. [73] But while the post-translational modification process in itself does not offer many clues as to the functional properties which are conferred on the protein by the attachment of a GPI anchor, the unusual physical disposition and dynamic behavior of these proteins is evident almost immediately following their synthesis, and suggest perhaps that the colligative properties of GPI-anchored molecules may be essential for characterizing their function on the cell membrane.

From the biophysical standpoint alone, the discovery of a molecule which is part-protein, part-saccharide, and part-lipid, is interesting since it introduces an entirely new structural element into the architecture of biological membranes. Based on the precepts set forth by fluid-mosaic model of the lipid bilayer [74], one might intuitively expect that a protein which is GPI-anchored would freely diffuse across the membrane surface, essentially as a typical phospholipid molecule. In our own published work [75], we used intensified video microscopy and fluorescent polystyrene spheres of 20~30 nm diameter, to show that the GPI-anchored molecules FcγRIIIB and DAF, incorporated into artificial supported bilayers composed of egg lecithin: cholesterol (80:20), and attached to the fluorescent beads via biotinylated 3G8

or IV.3 antibodies coupled to streptavidin covalently linked to the surface, exhibit a mean lateral diffusion $\langle D \rangle = 0.56$ and $0.25\ \mu m^2/sec$, respectively.

While these values are approximately an order of magnitude higher than corresponding values for typical membrane-spanning proteins ($\langle D \rangle \leq 0.01\ \mu m^2/sec$), they are significantly lower than the diffusion coefficient measured for fluorescent-conjugated phosphatidylethanolamine (PE) using the method of fluorescence recovery after photobleaching (FRAP), with $\langle D \rangle \approx 1.3\ \mu m^2/sec$. The slower diffusivity of the bead-attached GPI-anchored proteins may be the result of drag imposed by the microspheres, since the diffusivity of PE with similarly attached fluorescent beads was also estimated to be $\langle D \rangle \approx 0.26\ \mu m^2/sec$.

Perhaps not surprisingly, the behavior of GPI-anchored proteins in natural biological membranes appears more complex. Hannan and coworkers also used FRAP techniques to show that newly synthesized GPI-anchored proteins were initially clustered and immobile upon delivery to the membrane surface of MDCK cells, but became randomly diffusible after maturation. [76] Jacobson and colleagues used chimeric transmembrane and GPI-anchored transfectants to demonstrate that molecular interactions of the extracellular domain were at least as important as the lipidic moiety in determining the lateral mobility of GPI-anchored proteins. [77] While these studies alone are insufficient to characterize a functional role for glycolipid-linked membrane proteins, the observation of limited lateral mobility of these molecules in biological membrane (compared to that in artificial supported bilayers), strongly suggests that GPI-anchored proteins physically

interact with other membrane components, which may allude to their physiological role.

The disposition of GPI-anchored proteins within cells is also peculiar and has led to some intriguing findings. While normally restricted to the outer leaflet of the plasma membrane (or equivalent subcellular compartment in intracellular vacuoles), newly synthesized GPI-anchored proteins in polarized kidney or intestinal epithelial cells are shown to be further confined and directly targeted to the apical surface. [78] To identify the molecular determinants of this targeting, recombinant DNA methods were used to append the peptide sequence signaling for GPI attachment (taken from decay accelerating factor (DAF)), to the C-terminal domains of both a basolateral marker protein (herpes simplex glycoprotein D), and a regulated secretory protein (human growth hormone). The attachment to GPI was thereby shown to be sufficient to target the delivery of these fusion proteins to the apical surface of transfected Madin-Darby canine kidney (MDCK) epithelial cells. [79]

To further characterize the process which directs GPI-anchored proteins to the apical surface, Brown and Rose presented intriguing evidence in support of an association between sphingosine-based lipids and GPI-anchored placental alkaline phospatase (PLAP), which forms after the protein is transported to the intracellular Golgi apparatus. [80] With the notable absence of protein markers for the basolateral membrane, GPI-anchored proteins such as PLAP, together with glycosphingolipids were found to be specifically co-precipitated in low-density, detergent-insoluble complexes isolated from transfected MDCK cells that were lysed in cold, nonionic Triton

X-100. These detergent-insoluble structures provided the first demonstration of a hypothesis proposed by Simons and colleagues [81, 82], that GPI-anchored proteins and glycosphingolipids form specialized microdomains which sort into apically-directed targeting vesicles.

These findings, however, do not resolve the basic topological issue of signaling across the membrane bilayer by GPI-anchored molecules. Despite their co-localization in the Golgi complex, GPI-anchored PLAP and any associated glycosphingolipids remain shielded from physical contact with the cytoplasm proper, and hence cannot directly communicate their whereabouts to the cytosolic machinery responsible for directing vesicular traffic. However, Lisanti and colleagues, focusing more closely on the composition of the Triton-insoluble complexes relative to that of bulk membrane from MDCK cells, observed a pronounced enrichment not only in PLAP and sphingolipids, but also in other specific membrane molecules which formed what superficially resembled a transmembrane signaling assembly. Besides co-purifying other GPI-anchored proteins and cholesterol in the detergent-resistant fraction, Sargiacomo and coworkers identified membrane-associated molecules that either traverse the lipid bilayer (e.g. caveolin) or are oriented towards the cytoplasmic surface -- including small or heterotrimeric GTP-binding proteins, annexin II (a phospholipid binding protein involved in exocytic processes), and c-*yes* (a *src* family kinase). [83] Because of the distinct possibility that the co-purification of these molecules was merely an artifact of detergent cell lysis, the report fell short of claiming that these complexes represented actual microdomains isolated from the cell membrane.

Nevertheless, the identification of a group of molecules associated with GPI-anchored proteins which (at least theoretically) provided a framework for transmembrane signaling, led others to investigate the functional relevance of these detergent-insoluble membrane complexes. The involvement of caveolin was particularly intriguing for Rothberg and colleagues, who were investigating the mechanism of 5-methyltetrahydrofolate uptake by a GPI-anchored receptor. The endocytic process (which was termed *potocytosis*, [84]), was shown to involve specialized non-clathrin coated membrane pits called caveolae or *tiny caves*, which were identified by the presence of a 22 kDa transmembrane surface marker protein thus called *caveolin*. [85] It was shown that while individual folate receptors were randomly distributed over the membrane surface, clusters of the GPI-anchored receptor were distinctly localized within caveolin-coated invaginations, suggesting that caveolae represented a novel endocytic pathway for aggregated GPI-linked folate receptors. [86] Having showed previously that the cholesterol content in the membrane affected the clustering of the folate receptor, as well as the formation of the caveolar coat [85, 87], Rothberg and coworkers postulated that the membrane complexes resistant to solubilization by Triton X-100 might in fact be related to caveolae.

Since neutrophils do not express caveolin, it is doubtful whether the model of folate receptor-mediated potocytosis is relevant to signaling by FcγRIIIB. However, in a system much more closely related to neutrophils, GPI-anchored molecules have been shown to play a particularly active role in signaling. T lymphocytes are known to express large numbers of GPI-anchored proteins such as DAF [88], Thy-1 [89-91] and T cell activating protein (or TAP, a product of the LY-6 multigene locus [92]). Furthermore, upon

antibody crosslinking of any one of these proteins, T cells are induced to proliferate, especially when co-stimulated with phorbol esters. [88] The process of T lymphocyte activation by crosslinked GPI-anchored proteins was observed to bear striking resemblance to that seen when the T cell receptor (which is also a multichain antigen receptor containing ITAM modules) binds to major histocompatibility (MHC) class molecules on antigen presenting cells, and thus led to investigations into the involvement of TCR in signaling by GPI-anchored proteins.

Several lines of evidence demonstrated that expression of functional TCR/CD3 is absolutely necessary for stimulation of T cells by crosslinked GPI-anchored proteins. The downregulation of TCR expression induced by mAb crosslinking in normal T-cells, for example, impaired subsequent stimulation with anti-TAP mAb. [93] Similarly, a murine T cell line was used to demonstrate that antibody crosslinking of TAP triggers a proliferative response, as measured by [^{3}H]-thymidine uptake, and that this response is abrogated in TCR/CD3-negative deletion mutants. [94] Phorbol myristate (PMA, which stimulates protein kinase C) and calcium ionophore (which augments Ca^{2+} entry into the cytoplasm), were used to demonstrate that the signaling machinery downstream of TCR/CD3 was still functionally intact in these receptor knockout mutants, suggesting that the defect in signaling by GPI-linked TAP was directly related to the absence of functional TCR/CD3. Finally, specific deletion of the TCR-α and β chains, which contain the receptor binding domains, but not the signal transducing ITAM (**Figure 1**), did not affect the $[Ca^{2+}]_i$ transients induced by Thy-1 crosslinking, but inhibited Thy-1 induced interleukin (IL-2) production. Restoration of TCR-α and β gene expression in the TCR mutants also led to the recovery of Thy-1 activity

in stimulating IL-2 production, thus offering definitive proof that the T cell receptor is the requisite transmembrane signaling element for Thy-1. [95]

An important inference to be made from these collective studies is that the glycan phosphatidylinositol anchor itself, the only common element among the different GPI-linked proteins, must contain the essential signaling moiety within its structure. [93, 96, 97] While the complete chemical structure has been determined for only a subset of known GPI-anchored molecules -- including protozoal variant surface glycoprotein (VSG, [98]), Thy-1 from rat brain [99], acetylcholinesterase (AChE, [100]) and the complement regulatory protein, CD59 [101], both from human erythrocytes -- several interesting features found in the structure of GPI (shown schematically in **Figure 2**), suggest possible mechanisms for their function. Consequently, two general models of signaling by GPI-anchored receptors have emerged, which propose that these proteins communicate with transmembrane elements through interactions with either the glycan or lipid component of the GPI-anchor. [102, 103] These models will be introduced later and discussed in the context of our experimental findings.

EXPERIMENTAL DESIGN

The analysis of signaling by the Fcγ receptor system, and in particular the investigation of FcγRIIIB function, ultimately required that most experiments be performed on primary human neutrophils, since we found no cultured cell line which adequately expressed *and* supported functional GPI-anchored Fcγ receptors in preliminary screenings. Consequently, the experimental approaches for this study were greatly limited by technical and biological constraints imposed by these primary cells. Mature PMNs are terminally differentiated and relatively short-lived, with an average circulation time of approximately 8 hrs in the bloodstream. [45] In practice, neutrophils are viable for 4-5 hrs after venipuncture extraction, leaving a rather short window of time to perform experimental manipulations and assays. The cellular machinery of neutrophils is stripped down to the components which are essential for defending the host against foreign pathogens, hence they are virtually depleted of endoplasmic reticulum and ribosomal material. [45] This further rules out the possible application of conventional molecular biological methods to manipulate the FcγR signaling pathway in this system.

Finally, PMNs are among the most notoriously difficult primary cell types to handle in the laboratory. For instance, the expression of surface antigens (such as FcγRIIIB, but not FcγRIIA) on neutrophils may be perturbed during the isolation of these cells from whole blood by density gradient centrifugation. [104] PMN activation is also known to occur by contact with borosilicate glass or polystyrene. Furthermore, even in the absence of outside stimuli, neutrophils can be rendered unusable by spontaneous homotypic aggregation while in suspension. Paraformaldehyde fixation of specimens for

optical microscopy is also problematic, since neutrophils are particularly susceptible to a poorly characterized phenomenon of membrane "blebbing" [105], which significantly hinders the accurate analysis of the surface distribution of fluorescence-labeled FcγR. Moreover, neutrophil reactivity appears to vary greatly between healthy individuals, and may depend also on factors such as age, sex, smoking habits [106], etc... For many of the larger-scale studies which are described, the only solution for obtaining consistent and reliable results is by exhaustive iteration of procedures which are refined by trial-and-error, as well as by drawing blood samples from the same individual (usually myself) for the duration of a particular experiment.

The spectroscopic measurement of neutrophil activation by means of the $[Ca^{2+}]_i$-sensitive, fluorescent indicator Indo-1 is advantageous, not only because cytosolic free calcium is intimately involved with FcγR signaling, but also because the technique requires a minimum of preparatory cell manipulation, and the results are rapidly obtained and immediately interpretable. Similarly, the visualization of FcγR on the surface of living and activated neutrophils by immunofluorescence microscopy is essential for gaining an immediate understanding of the GPI-anchored receptors' behavior, and for helping to formulate a proposed mechanism of their function.

METHODS & MATERIALS

Cells

A murine macrophage cell line, $P388D_1$, was transfected with wild type or mutant human FcγRIIA cDNA as described. [10] The transfected $P388D_1$ cell lines with wildtype FcγRIIA (designated PW16) and mutant truncated receptors (Δ264 and Δ233) express 1.1-1.8 x 10^6 receptors per cell. The $P388D_1$ line was selected as a transfection host to enable analysis of FcγRIIA mutants without competition from endogenous FcγRIIA. Large quantities (~10^9 cells) of human neutrophils were isolated from buffy coat (Leukopac) preparations obtained from the Blood Donor Center of the Mount Sinai Hospital, and collected from the 1.119 g/ml interface of a two-step Histopaque (Sigma, St. Louis MO) density gradient, washed, and subsequently resuspended in Dulbecco's Modified Eagles (DME) medium containing 2% heat-inactivated fetal calf serum (FCS; Sigma) and 20 mM HEPES, pH 7.4. Alternatively, smaller scale PMN preparations (~10^7 cells) were isolated from whole venous blood by a one-step differential centrifugation protocol using Polymorphprep™ (Gibco BRL, Gaithersburg MD) solution, and held in M2 incubation buffer (150 mM NaCl, 20 mM HEPES, 1 mM $CaCl_2$, 5 mM KCl, and 10 mM glucose, pH 7.55; ref. [107]) at room temperature (rt) unless otherwise noted. In either protocol, contaminating erythrocytes were removed by 30 sec hypotonic lysis.

Derivatization of antibodies

Monoclonal mouse anti-FcγRIIIB 3G8 was obtained from Rhone-Poulenc (France); while anti-FcγRIIA mAb was purified from the culture supernatant of a IV.3 hybridoma (American *Type Culture* Collection, Rockville MD) by passage over Protein A-sepharose. Fab fragments were

prepared by digestion of whole IgG with 10 μg/ml activated papain (Sigma) for 4 hrs at 37°C. Cleaved Fc fragments and undigested IgG were removed from solution by immunoprecipitation with immobilized Protein G (Pierce Chemical Co., Rockford IL). Amine reactive fluorophores (Texas Red, rhodamine, and fluorescein isothiocyanates or succinimidyl esters; Molecular Probes, Eugene OR) were conjugated to Fab in 0.15 M bicarbonate buffer, pH 8.5, for 4 hrs at 4°C or 1 hr at rt, according to the suggested protocol. After blocking further conjugation with 0.15 M hydroxylamine, unreacted probe was removed by passage of the reaction mixture over a G-25 Sepharose column. Absorbance measurements showed typical conjugation ratios of 2-3 fluorophores/Fab.

Biotinylated probes were prepared by incubating ~1 mg of antibody dissolved in 1ml of bicarbonate buffer (pH 9.2) with 1.2 mg sulfo-NHS long chain (LC) biotin (Pierce) for 2 hrs on ice. Excess unreacted LC biotin was separated by passing the reaction mixture through a G-25 Sepharose column. For all derivatized antibody probes, the concentrations used to saturate labeling of FcγRIIA or FcγRIIIB on neutrophils in suspension ($\leq 10^7$ cells/ml) were approximately 2 μg/ml of IV.3 Fab and 5 μg/ml of 3G8 Fab, respectively.

Calculation of $[Ca^{2+}]_i$ from Indo-1 fluorescence ratio

In contrast to calcium probes whose fluorescence intensity changes upon binding of Ca^{2+}, Indo-1 is a UV excitable indicator derived from the Ca^{2+} chelator BAPTA [108, 109], which exhibits a spectral shift in the maximum of fluorescence emission from ~475 nm in calcium-free conditions to ~400 nm when the probe is saturated with Ca^{2+}. The ratio of the

fluorescence intensity measured at two emission wavelengths (Em 405/485, for instance) is therefore directly related to $[Ca^{2+}]_i$, and more importantly, is independent of cell thickness, and the concentration of dye within the cell, as long as it is below levels at which Indo-1 may begin acting as a calcium buffer. Indo-1 is particularly suited to measure intracellular calcium, since its dissociation constant for calcium (K_d~300 nM) matches the physiological range normally observed in neutrophils.

The ratio **R** of fluorescence intensity at the short wavelength ($F\lambda1$), divided by the intensity at the long wavelength ($F\lambda2$), can be used to calculate $[Ca^{2+}]$ by the following equation:

$$[Ca^{2+}] = K_d Q \frac{(R - R_{min})}{(R_{max} - R)}$$

where $\mathbf{R_{min}}$ and $\mathbf{R_{max}}$ are the ratio values measured at zero and saturating Ca^{2+} conditions, respectively; and **Q** is a sensitivity parameter defined as $(F\lambda2)zero/(F\lambda2)sat$, where the fluorescence intensities are measured at the long wavelength under Ca^{2+} free ($(F\lambda2)zero$) and saturating Ca^{2+} conditions.

Calcium spectroscopy

Neutrophils in suspension (3-6 x 10^6 cells/ml) were incubated with 1.5 μM of the calcium indicator, Indo-1 AM (acetoxymethyl ester, Molecular Probes), and unconjugated 3G8 or IV.3 Fab for 20-30 min (rt). Where indicated, cells were also incubated with wortmannin (Biomol Research, Plymouth Meeting, PA) during this interval. After incubation, the neutrophils were washed by pulse centrifugation and resuspended to densities of 0.5-2 x 10^6 cells/ml, either in M2 incubation buffer or in a balanced salt solution (BSS: 135 mM NaCl, 4.5 mM KCl, 5.6 mM glucose, 0.5

mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.4), and placed in a 1 cm pathlength quartz or acrylic cuvette. For inhibition studies, N,N-dimethylsphingosine (Biomol Research) was introduced to the labeled cells at the final resuspension step, in the presence of 0.1 mg/ml serum-free albumin to facilitate incorporation of the hydrophobic reagent.

Time-dependent Indo-1 fluorescence was recorded using a laboratory-modified, computer-controlled spectrofluorometer (SLM, Urbana IL) capable of simultaneous digital acquisition at two emission wavelengths. Excitation at 355 nm and emission at 390 nm were selected by monochromators, while the long wavelength Indo-1 emission band was observed through L-37 and Y-44 glass filters (Hoya Corp. USA Optics Div. San Jose CA). Neutral density filters were used to attenuate the excitation light intensity in order to minimize photobleaching of the probe.

Crosslinking of Fab-labeled FcγR was initiated by adding $F(ab')_2$ goat anti-mouse IgG (GαM; Jackson Immunoresearch, West Grove, PA) at a concentration of ~30 μg/ml. (**Figure 3**) As a positive control and to assess cell viability, 100 nM fMet-Leu-Phe (fMLP or chemotactic peptide; Sigma) was added to the cell suspension after the fluorescence ratio had returned to the baseline level. After background correction, the fluorescence intensity values were converted to intracellular calcium concentration, as described above. (also see ref. [108]) Fluorescence ratio values corresponding to saturating and calcium-free conditions, respectively, were determined by adding the calcium ionophore 4-bromo-A23187 (10 μM; Sigma) to the cell suspension, followed with 50 mM EGTA. A K_d value of 300 nM Ca^{2+} for Indo-1 at room temperature was used. [110]

Frustrated phagocytosis

Proteins were covalently coupled to glass as previously described.[111] Briefly, glass Petri dishes or coverslips were acid-washed with Chromerge® (VWR Scientific), rinsed thoroughly with distilled water, dried, and derivatized with 3-aminopropyltriethoxysilane (Sigma) for 4 min at rt. After rinsing with phosphate buffered saline (PBS), the dishes or coverslips were incubated with 0.25% glutaraldehyde (Sigma) for 30 min, rinsed, and then incubated for 1 hr with 5 μg/ml of GαM in 0.1 M sodium carbonate (pH 10). After final rinsing with PBS, residual reactive sites were quenched with 2% FCS. Cells suspended in DME with 2% FCS and 20 mM HEPES were then plated onto the coated glass at a density ~ 1 x 10^6 cells/6 cm diameter dish, and allowed to equilibrate for 20 min at 37°C. Where indicated, wortmannin was added 10 min prior to cell activation. Finally, Fcγ receptors were ligated to the coated glass by addition of IV.3 or 3G8 Fab (final concentration, 1-5 μg/ml) and the cells were incubated for various time intervals at 37°C. (ref. **Figure 4**)

For quantitative analysis of the phagocytic response by image-based cytometry, the adherent cells were fixed with 0.2% glutaraldehyde in PBS and then stained for 15 min with 0.2% Coomassie Blue R-250 dissolved in 20% methanol, and washed repeatedly with 5% acetic acid. Finally, the coverslips were quickly drained, air-dried, and mounted on slides with glycerol for observation. Digital images were acquired using a modified Zeiss Axiovert microscope equipped with a 10X/0.25 NA Achrostigmat or a 40X/0.75 NA water immersion objective and a cooled scientific CCD camera (OMA Vision, EG&G PARC, Princeton NJ). To further enhance the image contrast provided by the Coomassie stain, 560nm transillumination light was selected by a 40nm

bandpass interference filter (Omega Optical, Inc., Brattleboro, VT). The digitized image fields were analyzed automatically using the Image-1 software package (Universal Imaging, West Chester, PA) according to the following procedure: After applying a flatfield correction to minimize the image distortions due to non-uniformities in illumination and pixel sensitivity, and a median filter to reduce noise, each image was intensity-histogram thresholded to distinguish the opaque cells from the bright background, and the individual projected cell areas were determined in terms of number of pixels. [112] The absolute size of the image pixel was calibrated by use of an objective micrometer. Cell fragments and unresolved aggregates were excluded from analysis on the basis of their size or irregular shape.

FcγR internalization assay

^{125}I-radiolabeled probes were prepared from F(ab')2 fragments of rabbit anti-goat IgG (RαG; Jackson Immunoresearch) by incubating 100 µg antibody (1 mg/ml in PBS) with 15 µl ^{125}I (~100 µCi/ml) in glass tubes pre-coated with 1 µg iodogen (Pierce) for 5 min on ice. The reaction was stopped with 100 µl saturated tyrosine solution, and the conjugated antibody was purified by passage through a Sepharose G-25 column. Isolated neutrophils in suspension (1 x 10^6 cells/ml) were labeled in triplicate with 3G8 or IV.3 Fab (and preincubated with wortmannin, where indicated), transferred to thin-walled microtubes and held at 4°C on a programmable thermal controller (PTC-100) or Minicycler™ (MJ Research, Inc.; Watertown MA). After addition of GαM (30 µg/ml), the samples were warmed to 37°C for 0, 2 or 10 min to allow FcγR crosslinking and receptor internalization. Further membrane traffic was stopped by returning the samples to 4°C, and the cells were gently pelleted and washed by resuspending in cold buffer containing 5

μg/ml ^{125}I-conjugated RαG. (**Figure 10**) After 20 min, the labeled cells were spun down and washed twice in 15-fold volumes of cold PBS containing 5% FCS. The pellets were finally resuspended in 100 μl PBS, and the radioactivity measured using an automatic gamma counter (1271 Riagamma; LKB Wallac, Finland).

Incorporation of ITAM peptidomimetics by hypotonic shock

A peptide matching the 25 a.a. sequence bracketing the ITAM module of human FcγRIIA (NH_2-ADGGY**MTLNPR**APTDDDKNIY**LTLA**-$CONH_2$; FW: 2725 (+120)) was synthesized at the Protein Core Facility of the Mount Sinai School of Medicine. For use in control experiments, a second peptide was also prepared with substituted phosphotyrosine residues (NH_2-ADGGY**(p)MTLNPR**-APTDDDKNIY**(p)LTLA**-$CONH_2$; Fw: 2885 (+240)). Loading of the cells with peptide was carried out according to the procedure described by Maxfield and coworkers. [113] Briefly, stock solutions containing 20 mM peptide in M2 buffer and pH corrected to 7.6, were prepared beforehand. Following the procedure previously described for spectrofluorimetric calcium assay, aliquots of freshly isolated human neutrophils in suspension (3 x 10^6 cells/sample) were incubated for 40 min at 37°C, with 1.5 μM Indo-1 AM, 5 μg/ml IV.3 Fab, and 10 mM phosphorylated or unphosphorylated ITAM peptide (where indicated). The labeled and loaded neutrophils were subsequently pelleted by pulse centrifugation, and osmotically shocked for 30 sec in 900 μl distilled H_2O before restoring to isotonic medium with an additional 100 μl of 10 x PBS. The cells were then washed and transferred to suspension (10^6 cells/ml) in 1 cm-pathlength acrylic cuvettes for analysis.

To determine the efficiency of peptide loading by this method, mock trials were performed using FITC-dextran conjugate (avg. MW ~ 19,500; Sigma) in place of exogenous ITAM peptide. After hypotonic shock, the loaded neutrophils were washed three times by repeated centrifugation, resuspended in buffer, and the fluorescence intensity was measured in a spectrofluorometer. The concentration of internalized FITC dextran was estimated to be (at most) 5% of the concentration of the incubating solution, based on comparison with the fluorescence of calibration solutions of known FITC-dextran concentration, and on reasonable estimates of total cell volume.

Immunofluorescence/Confocal Microscopy

The distribution of Fcγ receptors on living cells was visualized by several methods. Isolated human neutrophils were labeled with either rhodamine-3G8 or fluorescein-IV.3 Fab, transferred to suspension in custom microtiter wells with a coverslip bottom, and viewed on a modified Zeiss Axiovert microscope equipped with Plan-Neofluar 100X/1.30 NA oil immersion objective, 100W xenon arc illumination, and optical filter sets for rhodamine and fluorescein (Omega Optical). Upon direct crosslinking of FcγR by addition of 30μg/ml GαM, time-dependent sequences documenting the kinetics of receptor aggregation were acquired using the OMA Vision CCD camera.

To investigate the co-localization of FcγRIIA and FcγRIIIB, neutrophils were labeled both with fluorescein-IV.3 and biotin-3G8 Fab. The 3G8 Fab-bound receptors were then crosslinked in suspension with 10 μg/ml Texas Red streptavidin (Molecular Probes, **Figure 16**) After allowing the labeled receptors to aggregate, the cells were centrifuged onto cleaned glass slides

(Cytospin 2, Shandon Southern Instruments, Inc.; Sewickley PA) and promptly fixed in ice cold methanol. The samples were air-dried and mounted in glycerol containing 1 mg/ml of the anti-fading agent, 1,4-phenylenediamine (Sigma-Aldrich, Milwaukee WI), and adjusted to pH 8.0. Fluorescence photomicrographs were taken on an Olympus BX60 system microscope, equipped with a UPlanFI 100X/1.30 NA oil immersion objective and with 100W mercury arc illumination. Exposure settings for 35 mm slide film were controlled automatically (PM-30 Exposure Control Unit, Olympus Instruments). Control samples containing one label only, were used to verify that the contribution of Texas red fluorescence to the signal detected through FITC optics, and vice versa, was not significant.

Confocal laser scanning microscopy was performed at the MSSM-CLSM core facility, supported with funding from NIH shared instrumentation grant (1 S10 RR0 9145-01) and NSF Major Research Instrumentation grant (DBI-9724504). Labeled cells, cytospun on glass, were imaged with a Leica confocal laser scanning microscope (CLSM) equipped with a krypton-argon laser source, a Plan Apo 100X/1.3 NA oil immersion objective, and filter sets optimized for fluorescein and rhodamine detection. Pinhole settings were adjusted to obtain optical sections of 0.5 μm thickness.

Neutrophil membrane labeling with DiI

Stock solutions of tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes) with various methylene chain lengths (C_{12}, C_{16}, and C_{18}) were prepared in methanol (~200 μg/ml). To improve DiI solubility in aqueous solution, isolated neutrophils were resuspended in 1 ml iso-osmotic mannitol (3×10^6 cells/ml), prior to the addition of fluorescein-3G8 Fab (5

μg/ml) and 3 μl of stock DiI solution, where indicated. After 20 min incubation at rt, the cells were washed with M2 incubation buffer containing 0.1 mg/ml serum albumin, and transferred to droplets on glass coverslips precoated with silicone (Sigmacote®, Sigma), for direct observation by fluorescence microscopy.

Determination of RET using far-field spectroscopic methods

To determine whether FcγR co-localization occurs on the nanometer distance scale, below the optical resolution of both wide-field and confocal microscopes, neutrophils were initially labeled with rhodamine-conjugated 3G8 Fab and triggered with 30 μg/ml GαM. Approximately 5~7 min after FcγRIIIB crosslinking, the cells in suspension were fixed briefly with 3% cold paraformaldehyde, washed and subsequently labeled with 5 μg/ml fluorescein-IV.3 Fab in the presence of excess unconjugated 3G8 Fab (to saturate any free GαM binding sites). The double-labeled cells were finally mounted on glass slides in glycerol with 1,4-phenylenediamine (1 mg/ml). Control specimens singly labeled with either fluorescein IV.3 or rhodamine 3G8 Fab were also prepared.

FcγR caps were examined by using an experimental setup constructed by Trautman and colleagues of AT&T Bell Laboratories (now Lucent Technologies, Inc.) at Murray Hill NJ, to carry out near-field optical microscopy and spectroscopy measurements with single-molecule sensitivity. [114, 115] For our experiments, rather than using the near-field probe, we opted for a simpler far-field, epi-illumination optical arrangement (illustrated in **Figure 18**), in which fluorophores were excited by a tightly focused beam of

light (488 nm) from a picosecond pulsed argon ion laser, and observed through a standard 1.25 NA/100X oil immersion objective lens.

The emitted fluorescence was directed onto one of two alternative paths: (1) light brought in via optical fiber, was dispersed by a monochromator /spectrograph, onto a liquid nitrogen-cooled CCD camera, which allowed measurement of the fluorescence emission spectrum; (2) the emitted light, after passing through an interference filter (515 nm/35 nm FWHM for fluorescein, 550 nm/30nm FWHM for rhodamine), was focused onto a low-noise avalanche-photodiode detector, operating in single-photon counting mode, which enabled both signal integration for the acquisition of scanned images, as well as measurement of the fluorescence decay, by means of time-resolved spectroscopic approaches.

In our particular experiment, the fluorescein-IV.3 Fab within a Fcγ receptor cluster were specifically selected for analysis of RET. The time-resolved nanosecond fluorescence decays measured in the absence and presence of rhodamine-labeled 3G8 Fab acceptors yielded fluorescein lifetimes τ_D and τ_{DA}, from which the efficiency of RET could be calculated. (see Results for quantitative methods.)

RESULTS I: *FUNCTIONAL EVIDENCE*

FcγRIIA tethering induces frustrated phagocytosis

Response to FcγR ligation of transfected P388D1 cells

Following reports about the T cell responses to crosslinking ligands attached to a cell-impermeant matrix [116], our laboratory developed a similar assay to determine whether signaling by FcγR is affected by the immobilization of crosslinking antibody to glass. [111] In contrast to untreated borosilicate glass, the glass surface derivatized with F(ab')2 goat anti-mouse IgG (GαM) did not by itself cause adherent phagocytes to spread. Introduction of IV.3 Fab, however, led to the direct coupling of FcγRIIA on the macrophage cell surface, to the F(ab')2 fragments of anti-mouse IgG which were covalently attached to the glass (schematized in **Figure 4**), resulting in the dramatic spreading and flattening of these transfected cells compared to the untransfected control. (**Figures 5A,B**) Maximal spreading was seen after 10 min and persisted for at least 4 hrs after ligation. Equivalent results were obtained when cells were plated on glass similarly coated with streptavidin and triggered with biotinylated-IV.3 Fab (not shown).

The morphological changes observed in the PW16 cells are dependent on signaling mediated by FcγRIIA, and not simply due to the physical ligation of the receptor to the substrate. P388D1 cells expressing the FcγRIIA mutants Δ233, in which the complete ITAM sequence is deleted, and Δ264, which lacks the carboxy-terminal YXXL motif, failed to spread under the same conditions which evoked a response in PW16 (**Figures 5C-F**), although the level of expression of the transfected FcγRIIA deletion mutants is comparable. The results of the quantitative image analysis of cell spreading, displayed in **Figure 6** as frequency histograms of projected cell areas, show that 10 min after

FcγRIIA ligation the area of over 85% of PW16 macrophages was greater than 200 μm^2/cell, whereas 85% of P388D1 cells expressing the Δ233 and Δ264 deletion mutants covered areas less than 200 μm^2/cell. This corroborates previous findings that the Δ233 mutant receptors appear to be completely non-functional, mediating neither tyrosine phosphorylation, Ca^{2+} flux, internalization of small complexes, nor phagocytosis. However, the removal of only the carboxy-terminal YXXL motif in the Δ264 FcγRIIA mutant preserves the normal endocytosis of small complexes, while abrogating phagocytosis of IgG-sensitized erythrocytes and Ca^{2+} release from intracellular stores. [10] The similar dependence of the two processes, phagocytosis of coated erythrocytes and the spreading response of cells on derivatized glass, on the presence of intracellular Ca^{2+} and on the intact expression of FcγRIIA ITAM, strongly suggests that this assay is an accurate measure of the phagocytic response mediated by FcγRIIA.

Phagocytic response of human neutrophils to FcγRIIIB and FcγRIIA ligation

Similar assays were performed on neutrophils freshly isolated from peripheral blood, and again, ligation of the transmembrane FcγRIIA to GαM-conjugated glass via IV.3 Fab was shown to result in dramatic cell spreading compared to the unstimulated control. (**Figure 7**) As observed previously in macrophages (**Figure 5G**), the neutrophil response to FcγRIIA ligation was blocked in the presence of the intracellular Ca^{2+} chelator BAPTA, which was introduced by preincubating PMNs for 30 min with 100 μM of its acetoxymethylester derivative (BAPTA-AM). Furthermore, since phagocytosis in neutrophils is absolutely dependent on tyrosine phosphorylation, we confirmed that the spreading response was also inhibited in PMNs preincubated for 30 min with 10 μg/ml of the protein

tyrosine kinase inhibitor, genistein. (results not shown) In contrast, ligation of the GPI-anchored FcγRIIIB to the GαM derivatized surface by means of 3G8 Fab was ineffective at stimulating morphological changes in the neutrophils. (**Figure 7**)

Wortmannin blocks FcγRIIA-mediated spreading response in phagocytes

Preincubation of either PW16 macrophages or human neutrophils with 10 nM wortmannin inhibited cell spreading upon ligation of FcγRIIA to the derivatized glass by means of IV.3 Fab. In order to obtain a quantitative estimate of the phagocytic response and its dependence on PI 3-kinase activity, we again used image-based cytometry to gather statistical data on the projected surface area of phagocytes stimulated by ligation of FcγRIIA. Dose-inhibition curves were obtained by measuring the phagocytic response as a function of wortmannin concentration in the preincubation medium. (**Figure 8**) Our assays indicate that the IC_{50} of wortmannin is approximately 2 nM in human neutrophils and approximately 23 nM in the transfected PW16 macrophages. While the IC_{50} estimated in neutrophils is in good agreement with the value previously reported for PI 3-kinase partially purified from guinea pig neutrophils[28], the ten-fold higher IC_{50} estimated in macrophages may reflect an enhanced ability of these cells to sequester or pump out wortmannin, or perhaps a lower intrinsic susceptibility of the murine PI 3-kinase toward the wortmannin inhibitor.

Antibody crosslinking of FcγR triggers intracellular calcium release

We note that the inhibition of frustrated phagocytosis by wortmannin corresponds to a similar inhibition observed in phagocytes incorporating the Ca^{2+} chelator BAPTA, suggesting that PI 3-kinase mediates phagocytosis by

modulating $[Ca^{2+}]_i$. In principle, the abrogation of intracellular calcium release by the presence of wortmannin would place PI 3-kinase upstream of the signaling components responsible for Ca^{2+} mobilization. We were therefore interested in conducting extensive spectrofluorometric assays of $[Ca^{2+}]_i$ in cells loaded with the ratiometric fluorescent indicator, Indo-1.

In neutrophils under normal control conditions, crosslinking of either IV.3 Fab-labeled FcγRIIA or 3G8 Fab-labeled FcγRIIIB by addition of GαM triggers rapid (< 2 min) and substantial increases in cytosolic free calcium, sometimes approaching 1 μM. Interestingly, we notice a slight but consistent lag in the induction time for calcium release by crosslinked FcγRIIIB compared to FcγRIIA. This phenomenon, which is evident in **Figure 9** (Graph I), may be relevant to the kinetics of signaling by the GPI-linked receptor. The $[Ca^{2+}]_i$ transients triggered by the Fcγ receptors did not reach magnitude of those observed in neutrophils exposed to nanomolar concentrations of the chemotactic peptide, formyl-Met-Leu-Phe (fMLP), which was added to the cell suspensions at the end of each assay as a standard control to ensure that the intracellular calcium release mechanism was still intact.

Wortmannin blocks FcγR triggered $[Ca^{2+}]_i$ transients in neutrophils

The release of calcium from intracellular stores triggered by crosslinking of either FcγR species is exquisitely sensitive to inhibition by wortmannin ($IC_{50} \approx 2$ nM), as illustrated by the dose response curves in **Figure 9** (Graph II). In contrast, wortmannin does not affect the $[Ca^{2+}]_i$ transients triggered by activated fMLP receptor, which mobilizes intracellular calcium via the release of IP_3 by phospholipase C-beta (PLC-β) activation. [44]

This suggests either that Ca^{2+} release in response to FcγR crosslinking is not mediated by the IP3 second messenger, or that the FcγR-mediated calcium mobilization pathway specifically involves PI 3-kinase activity upstream of IP3 production, perhaps by controlling access of PLC-γ to substrate.

Corresponding to the results found in the frustrated phagocytosis assay, the determination of $[Ca^{2+}]_i$ transients in PW16 macrophages triggered by antibody crosslinking of FcγRIIA showed a somewhat lower sensitivity to inhibition by wortmannin ($IC_{50} \approx 33$ nM) as compared to neutrophils. (**Figure 8**) Although the reason for this lower susceptibility is unclear, it is satisfying to note that the relative sensitivity to inhibition by wortmannin is consistent for each cell type and is independent of whether FcγRIIA-induced activity is measured by cell spreading, or by intracellular calcium release. These findings further reinforce the notion that the phagocytic response mediated by FcγRIIA absolutely requires the calcium second messenger.

Receptor mediated endocytosis is unaffected by wortmannin

While the internalization of FcγRIIA upon binding to immune complexes has been well documented, the fate of similarly crosslinked FcγRIIIB has not been characterized. As illustrated by the graphs in **Figure 11**, we observe that both 3G8 Fab-labeled FcγRIIIB and IV.3 Fab-labeled FcγRIIA are sequestered from the cell surface within 3 minutes of crosslinking at 37°C by soluble GαM (30 μg/ml), based on the decreased binding of ^{125}I-conjugated tertiary anti-goat $F(ab')_2$ to crosslinking GαM remaining on the neutrophil surface. (**Figure 10**) At concentrations sufficient to completely block FcγR-mediated calcium release and frustrated phagocytosis (≤ 100 nM), however, wortmannin did not affect receptor internalization. In PW16 macrophages

incubated with 100 nM wortmannin, we also did not observe any significant change in the rapid ($t \leq 2$ min) endocytosis of IV.3 Fab-labeled FcγRIIA crosslinked with GαM (data not shown). This parallels the results from the deletion mutants, in which the d233 macrophages are incapable of phagocytosis and intracellular Ca2+ release, but remain competent at endocytosis.

Dimethylsphingosine blocks FcγR mediated calcium

Our observation that wortmannin blocks the mobilization of intracellular calcium following FcγR stimulation, but not after fMLP receptor stimulation, suggests that neutrophil FcγR may utilize an entirely different Ca^{2+} release pathway, compared to that known for the G protein-coupled receptor. Following reports that sphingosine kinase is involved in FcεRI-mediated calcium release in rat basophilic leukemia cells [47], we performed initial assays to determine if DL-*threo*-dihydrosphingosine, which was shown to block the calcium flux in RBL cells, had a similar inhibitory effect on human neutrophils. We found, however, that the reagent was poorly soluble in aqueous solution, and difficult to administer to live cells in suspension. However, we obtained consistent results with N,N-dimethylsphingosine, another more soluble inhibitor of sphingosine kinase. As shown by the $[Ca^{2+}]_i$ traces in **Figures 12A,B**, preincubation of Indo-1 loaded PMNs with 1 μM dimethylsphingosine strongly inhibited the transient rise in $[Ca^{2+}]_i$ triggered by either FcγRIIIB or FcγRIIA crosslinking, without significantly affecting the $[Ca^{2+}]_i$ elevation triggered by fMLP. These results strongly suggest that the calcium transients observed following either FcγRIIA or FcγRIIIB ligation are mediated by the activation of a sphingosine 1-phosphate

pathway, rather than via the more conventional IP3-dependent release mechanism.

We also determined an approximate dose dependence of FcγR-mediated $[Ca^{2+}]_i$ transients to inhibition by N,N-dimethylsphingosine. During the course of these studies, we observed that the extent of inhibition by a given concentration of the compound was affected by variations in the density of neutrophils in the incubation suspension (1~2 x 10^6 cells/ml). We believe that this may be due to strong partitioning of the sphingosine analogue into the cell membrane. Indeed, at bulk concentrations of dimethylsphingosine higher than approximately 10 μM, our measurements of Indo-1 fluorescence indicated that the neutrophil membranes had become permeabilized. (data not shown). Therefore, we have chosen to express the concentration of N,N-dimethylsphingosine in relation to the *density* of cells in suspension, which is proportional to the volume fraction of the membrane phase in the partitioning equilibrium. From the dose curve presented in **Figure 12C**, an $IC_{50} \approx 0.5$ nanomoles/10^6 (cells · ml^{-1}) can be deduced for inhibition of FcγR-mediated $[Ca^{2+}]_i$ transient by N,N-dimethylsphingosine.

Dependence of FcγRIIIB signaling on FcγRIIA function

Given the strong evidence that FcγRIIIB and FcγRIIA share convergent signaling pathways, I attempted to directly test the hypothesis that signaling by FcγRIIIB actually requires the participation of FcγRIIA. The most straightforward means to verify this hypothesis would be to use antisense cDNA or other molecular biological approach to knock out the expression of the transmembrane receptor. Unfortunately, as previously mentioned,

neutrophils are not amenable to this approach. Less direct methods were therefore required to disrupt the signaling activity of FcγRIIA, in order to investigate the residual cell activation upon crosslinking FcγRIIIB. However, the two experiments described here yielded inconclusive results, and will require further modification to be useful for future investigations.

Analysis of sequential FcγR crosslinking

The evidence of decreased ^{125}I-RαG binding to IV.3 Fab-labeled neutrophils crosslinked with GαM (previously shown in **Figure 11**), suggests that the surface density of FcγRIIA is significantly reduced for a period lasting from 2 to at least 10 min after initial antibody crosslinking. We have also shown from the observed $[Ca^{2+}]_i$ kinetics that, within this same time interval, neutrophils can recover from FcγR stimulation to baseline $[Ca^{2+}]_i$ levels and are responsive to triggering by a different second stimulus (usually fMLP). Based on these observations, the dependence of FcγRIIIB signaling on interaction with FcγRIIA could be inferred (at least in principle), by a relative decrease in the amplitude of the $[Ca^{2+}]_i$ response triggered by crosslinking FcγRIIIB during the interval when FcγRIIA is presumably depleted from the neutrophil surface.

The results of such an experiment are summarized in the panels shown in **Figure 13**. Isolated neutrophils were labeled with both biotinylated IV.3 Fab and unconjugated 3G8 Fab, loaded with the Indo-1 calcium probe, and transferred into cuvettes for spectrofluorimetric monitoring. Approximately 10 min after FcγRIIA crosslinking had been induced by the addition of 30 μg/ml streptavidin, GαM (also 30 μg/ml) was also added to the neutrophil suspension to trigger FcγRIIIB-mediated calcium release. As

expected, the crosslinking of FcγRIIA itself stimulated a brief $[Ca^{2+}]_i$ transient (**Fig. 13A**); but surprisingly, the subsequent rise in $[Ca^{2+}]_i$ following FcγRIIIB crosslinking was even larger in magnitude than that observed in control specimens labeled only with 3G8 Fab, in which FcγRIIIB was crosslinked without prior activation of FcγRIIA. (**Fig. 13B**)

To assess whether this apparent potentiation was caused by GαM cross-reacting with biotin-IV.3 Fab-labeled FcγRIIA crosslinked by streptavidin, I also performed the same experiment on cells labeled only with biotinylated IV.3 Fab. Under these conditions, the addition of GαM did not evoke an additional $[Ca^{2+}]_i$ response after that triggered by the initial crosslinking with streptavidin. (**Fig. 10C**)

For the sake of completeness, I also performed a set of experiments in which PMNs were labeled with biotinylated 3G8 Fab and unconjugated IV.3 Fab, and the order of FcγR crosslinking was reversed. The results, which are not shown, were exactly analogous to those described above, in that: (1) crosslinking FcγRIIA following activation of FcγRIIIB produced a larger $[Ca^{2+}]_i$ transient than that observed when FcγRIIIB was not initially crosslinked, and (2) addition of GαM did not further stimulate biotin-3G8 Fab labeled neutrophils which had been previously triggered with streptavidin.

Inhibition of FcγR signaling by exogenous ITAM peptide incorporation

The successful use of ITAM peptidomimetics to characterize the early events following T cell receptor activation [11], inspired me to adopt this technique to examine the mechanism of FcγR signaling in neutrophils. The rationale behind this approach is that the introduction of an exogenous

oligopeptide containing the ITAM sequence of FcγRIIA (or its bi-phosphorylated analogue) should specifically inhibit or at least significantly perturb signaling by FcγRIIA, by competitively binding cytosolic signaling molecules which would otherwise recognize the activated receptor.

An oligopeptide was synthesized at the Protein Core Facility of the Mount Sinai School of Medicine, which closely matched the 25 amino acid sequence bracketing the ITAM motif of FcγRIIA. For use as a control, the same peptide sequence was also prepared with phosphotyrosine substituted at both tyrosyl positions. Designing a method to reliably incorporate these peptidomimetics into viable neutrophils, proved to be the most problematic step in this experiment. While electroporation was shown to effectively permeabilize PMNs to diffusion of small peptides [117], the recovery of cell membrane integrity following such a procedure was not rapid enough to permit subsequent analysis of calcium ion flux by Indo-1 spectrofluorimetry. Therefore, I opted for a method successfully implemented by Maxfield and coworkers to incorporate peptide inhibitors into neutrophils. [113]

Unfortunately, the preliminary results which I obtained through $[Ca^{2+}]_i$ measurements were not encouraging. Even at the highest peptide concentrations which were practical, neither the unphosphorylated nor the phosphorylated ITAM peptidomimetics significantly perturbed the $[Ca^{2+}]_i$ transients stimulated by crosslinking FcγRIIA. (data not presented) Without this crucial result, I could not continue the investigation to test whether these synthetic peptides could alter signaling by the GPI-anchored receptor.

RESULTS II: *PHYSICAL EVIDENCE*

Surface distribution studies of FcγRIIA and FcγRIIIB

FcγR capping: "active vs. passive" response to crosslinking.

The surface distribution of FcγRIIA and FcγRIIIB was examined by immunofluorescence microscopy on live neutrophils labeled with fluorophore-conjugated IV.3 and 3G8 Fab. In cells labeled only with fluorescein-3G8 Fab, the GPI-linked FcγRIIIB is initially uniformly distributed over the membrane surface. Within 5~7 min of receptor crosslinking by GαM, however, the distribution of FcγRIIIB appears coalesced into polar caps, as illustrated by the image sequence in **Figure 14A**. Similar receptor capping is also displayed by crosslinked FcγRIIA. (data not shown) Although clustering of FcγR is immediately evident upon GαM crosslinking, the progression to large polar aggregates requires up to 10 min at room temperature. Compared to the kinetics of FcγR-mediated intracellular calcium release, the formation of the FcγR cap requires significantly longer time, and is probably not the event which initiates cytosolic signaling in the neutrophil. On the other hand, receptor capping may reflect the progression of intracellular signaling events (such as F-actin polymerization and cytoskeletal reorganization) which occur farther downstream of FcγR activation.

To illustrate that the Fcγ receptor capping reflects an active signaling process, as opposed to passive clustering in response to binding of multivalent ligand, we studied the evolvement of the surface distribution of human FcγRIIIB transfected into a rat basophilic leukemia cell line (RBL-6CD5), during crosslinking with GαM. As shown in the second pair of images in **Figure 14B**, the labeled FcγRIIIB appears initially distributed in patches

approximately 1-2 μm in diameter on the cell surface. Upon receptor crosslinking, these patches are observed to condense and perhaps fragment into smaller clusters with diameters equal to, or smaller than, the optical resolution of ~ 250 nm. In marked contrast to the results seen on neutrophils, the aggregation of FcγRIIIB on the surface of 6CD5 cells, even 20 min after receptor crosslinking, does not proceed further to form receptor caps. This correlates well with our observation that antibody crosslinking of FcγRIIIB in 6CD5 cells evokes little or no intracellular calcium response (data not shown), which may in fact be a prerequisite for the receptor capping process.

Wortmannin disrupts receptor cap formation

The inhibition of PI 3-kinase by wortmannin profoundly disrupts the aggregation of FcγR in the neutrophil. Shown in **Figure 15** are confocal images of PMNs labeled with only one probe, either rhodamine-3G8 or fluorescein-IV.3 Fab, and preincubated with 30 nM wortmannin, 5 min after crosslinking with GαM (as described in Methods). Instead of coalescing into large patches on the surface, aggregates of FcγRIIA and FcγRIIIB appear to be distributed in a dispersed pattern throughout the entire area subtended by the cell. The exact location of the receptor clusters with respect to the cell surface is difficult to ascertain. Given that the cells were cytospun before fixing, the FcγR aggregates may actually be on the surface, since the thickness of the flattened cells may be comparable to the depth resolution of the confocal microscope. Nevertheless, the localization of the fluorescent clusters within the cytosol would be in agreement with the results of our internalization assays, which indicate that wortmannin does not block Fcγ receptor internalization following crosslinking.

Co-localization of FcγRIIA with aggregated FcγRIIIB

Although FcγR co-localization is a natural consequence of binding to nonspecific immune complexes (which we demonstrate in **Figure 17C**), we were interested in determining if the lateral redistribution of FcγRIIA might also be affected by large-scale movement of the GPI-anchored isoform. Such a finding would more clearly demonstrate a physical association between the two receptor species. To investigate this possible effect using fluorescence microscopy, however, required a different labeling strategy from that used previously. Since both 3G8 and IV.3 mAbs are murine-derived, we were restricted from using polyclonal GαM to selectively crosslink 3G8 Fab-labeled FcγRIIIB, in the presence of fluorescein-conjugated IV.3 Fab bound to FcγRIIA. As a result, two different labeling schemes were developed, both of which enabled the visualization of the relative distribution of FcγRIIIB and FcγRIIA, at the same time permitting the selective ligation and crosslinking of FcγRIIIB.

The first technique involved an initial labeling of PMNs with rhodamine-3G8 Fab (only), followed by crosslinking of the labeled FcγRIIIB with GαM. Before the secondary addition of fluorescein-conjugated IV.3 Fab to probe for FcγRIIA however, the cells were fixed briefly to preserve the surface distribution of aggregated FcγRIIIB, and exposed to unconjugated 3G8 Fab to block further reactivity with the crosslinking GαM. The results obtained by this method were somewhat inconsistent, due primarily to the intervening step of paraformaldehyde fixation, which caused membrane blebbing and significantly altered surface FcγR distribution. (data not shown.)

To obviate these difficulties, we developed a one-step double labeling technique (described in the Methods section and shown in **Figure 16**) which uses Texas Red-conjugated streptavidin to specifically crosslink biotinylated 3G8 Fab-labeled FcγRIIIB, in the presence of fluorescein-IV.3 Fab bound to FcγRIIA. Although the experiment was substantially streamlined by adopting this strategy, the labeled neutrophils still required fixing to allow washing of excess fluorescent streptavidin. We chose therefore to cytospin the labeled cells onto glass slides, and to fix the cells in cold methanol, thus eliminating the artifacts of paraformaldehyde fixation entirely.

This second method yielded satisfactory results, as represented by the photomicrographs in **Figures 17A.1-3**, and confirmed using confocal microscopy (**Figure 17B**), both of which show that the distribution pattern of fluorescein labeled FcγRIIA overlaps with regions of aggregated FcγRIIIB, identified by Texas Red fluorescence. Because these cells were subjected to cytospinning, the resulting morphology is clearly flattened from the spherical form typically observed for live neutrophils in suspension. Again, because of the reduced thickness of the cells, it is impossible, even at the 0.5 μm depth resolution of the confocal setup, to establish whether the receptor clusters are situated on the cell surface or are internalized in vesicular structures, as we would expect from the results of our internalization assays. We confirmed that the observed punctate pattern of fluorescence truly represented the localization of labeled FcγR, by imaging unlabeled cells which were similarly prepared, and which exhibited no detectable autofluorescence. Furthermore, in cells which were cytospun and fixed within 1 min of addition of Texas Red streptavidin, both fluorescent-labeled probes appeared uniformly distributed over the area of the flattened cells, (not shown)

In other experiments, the complementary labeling and crosslinking combination was applied, i.e., using biotinylated IV.3 Fab and fluorescein-conjugated 3G8 Fab. In this case, we found that the co-migration of labeled FcγRIIIB into capped regions of crosslinked FcγRIIA was far less prominent (data not shown), perhaps due to the large excess of GPI-anchored FcγRIIIB with respect to the population of the transmembrane isoform.

Receptor capping vs. receptor internalization

The movement of FcγR which we have observed in living neutrophils, using immunofluorescence microscopy and internalization assay, represents a behavior which clearly is relevant to their physiological function. But while we demonstrate that fluorescent-labeled Fcγ receptors form polar caps on the cell surface within 5-7 min of crosslinking, we also show that more than 2/3 of both FcγRIIA and FcγRIIIB are sequestered from the surface following receptor crosslinking, with similar kinetics. These findings are not necessarily contradictory, but rather, may reflect two similar, yet distinct, biological processes which are both induced by crosslinking FcγR as we have done. The mechanistic differences which distinguish phagocytosis from receptor-mediated endocytosis, are presently unclear. However, differences in temperature may significantly affect the relative rates of these processes. In contrast to the rapid endocytosis which we observe at 37°C by internalization assay, at ambient temperatures (18-23°C) membrane traffic may slow down sufficiently to allow crosslinked FcγR to aggregate into large caps at the surface before a majority of the receptor becomes incorporated into the cell. Whether the capped receptors are truly exposed at the surface, or are already contained in vesicles just underneath the plasma membrane, is a detail which we have

not yet elucidated. Nevertheless, for purposes of this study, the physicial disposition of FcγRIIIB consistently parallels that of FcγRIIA, under all situations examined. This evidence alone is sufficient to suggest that the two receptor species are physically associated.

Analysis of the FcγR cap by fluorescence resonance energy transfer

Given the physical evidence from optical microscopy for an association between FcγRIIA and FcγRIIIB, I was interested in determining whether the co-capping receptors come into direct intermolecular contact. Due to the diffraction-limited resolution ≥ 0.2 μm, inherent to conventional optical systems, proximity on the molecular scale cannot be ascertained, even from perfectly overlapping fluorescence distributions.

In contrast, the method of fluorescent resonance energy transfer (RET) can be used to investigate molecular proximity on the nanometer distance scale. RET is the non-radiative mechanism by which an excited donor fluorophore (D) transfers its excitation energy to an appropriate acceptor molecule (A). Since this quantum-mechanical exchange occurs via dipole-dipole interaction, the rate of RET decreases with the inverse sixth power of the D-A separation distance, R. In practice, RET can be detected over distances comparable to the dimensions of biological macromolecules, and is characterized by the Förster radius, R_0, which is uniquely determined for a given donor-acceptor pair and is mainly determined by the extent of overlap between the D emission and the A absorption spectra. (R_0 for a fluorescein donor and rhodamine acceptor, for example, is approximately 5 nm.) At a separation distance of R_0, an excited fluorophore is as likely to emit a photon,

as it is to transfer its excitation energy to the nearby acceptor molecule. Under these circumstances, the steady state fluorescence intensity (F) and excited state lifetime (τ) of the donor fluorophore in the presence of the acceptor (subscript $_{DA}$) are reduced to half the values measured in the absence of A (subscript $_{D}$). The efficiency of RET (E_T) can readily be calculated from these measurements, by the following relation:

$$E_T = 1 - \left[\frac{\tau_{DA}}{\tau_D}\right] = 1 - \left[\frac{F_{DA}}{F_D}\right]$$

The process of RET contributes to the rate of depopulation of the excited state donors, which can be measured by a deviation in the steady state intensity of the donor fluorescence or by a shortening of the donor fluorescence lifetime. The practice of detecting RET by steady state fluorescence intensity (although used extensively by Petty and coworkers. [102, 118]) requires laborious calibration procedures to ensure accurate data, since fluorescence intensity is highly dependent on fluctuations in excitation intensity, probe concentration, as well as fluorophore photobleaching.

On the other hand, the excited state lifetime is an intrinsic property of the fluorophore, independent of concentration (within reasonable limits), optical pathlength and excitation intensity. For these reasons, we opted to measure RET by time-resolved fluorescence spectroscopy on clusters of labeled FcγR, using a novel experimental set-up developed by Dr. Jay Trautman (formerly at Bell Labs, Murray Hill NJ; [114, 115]), and following the procedure described in Methods & Materials. This technique allowed us to explore the use of an experimental setup, originally intended for near-field scanning optical microscopy (NSOM), for determining the distribution of fluorescent-labeled 3G8 and IV.3 mAbs with spatial resolution better than the

optical diffraction-limit and with single molecule sensitivity. In the context of our biological system, a close proximity (on the molecular scale) between FcγRIIIB and FcγRIIA could be inferred, by the detection of a decrease in the excited state lifetime of fluorescein-labeled IV.3 Fab bound to FcγRIIA (participating as RET donors), in the presence of rhodamine-labeled 3G8 Fab bound to FcγRIIIB (functioning as RET acceptors).

We performed such an analysis, using a novel experimental set-up (illustrated in **Figure 18**) which permits the simultaneous measurement of emission spectra and time-resolved decays of individual fluorescent clusters on the surface of fixed cells, as well as the acquisition of images by piezo-electric scanning of the excitation source over the cell area. Because the fluorescein and Texas Red fluorophores are unlikely to participate in RET, we had to resort to the original method of double labeling, which involved the intermediate paraformaldehyde fixation step. The series of composite diagrams in **Figure 19** show images of representative cells obtained with the far-field imaging system previously described, together with the corresponding fluorescence spectra and time-resolved decays measured from the indicated regions. Under all conditions, the fluorescence decays were complex, requiring at least two exponentials to achieve acceptable fits. From the experimental data which we collected, no definite change was observed in the average excited state lifetime of the fluorescein label in the presence of rhodamine acceptors.

While a shorter donor lifetime would have strongly suggested that the two species of Fcγ receptors were brought into close proximity, a lack of RET should not be taken as conclusive evidence of the absence of such interaction,

as this may simply result from the specific method of labeling the Fcγ receptors. Given the strong distance dependence and relatively short range of RET, the physical dimensions of the Fab fragments may contribute substantially to the distance separating donor and acceptor molecules, thus precluding the occurrence of RET.

Co-localization of DiI with FcγRIIIB

A previous report that various fluorescent lipophilic tracers, such as the cationic membrane probe DiI, were nonrandomly distributed with FcεRI in RBL cells [119], suggested that the properties of the lipid bilayer immediately surrounding certain membrane proteins might be significantly altered from those of bulk membrane. Such local properties might lead to the formation of discrete lipid domains on the plasma membrane enriched in specific proteins and other components. To determine whether such behavior was exhibited by FcγRIIIB in neutrophils, I performed preliminary experiments to observe the distribution of DiI, which is anchored to the membrane via two saturated, 16-carbon long ($C_{16:0}$) methylene chains, in relation to that of fluorescein-3G8 Fab-labeled FcγRIIIB. As illustrated by the fluorescence micrographs in **Figure 22**, the lipophilic probe is found to preferentially migrate with fluorescein-labeled FcγRIIIB, into the region of the GPI-anchored receptor cap induced by crosslinking with GαM.

Based on the structural analysis of acetylcholinesterase and CD59 from human erythrocytes, which has revealed an unusual, 22-carbon long, multiply-unsaturated acylation ($C_{22:4}$) of the GPI anchor at the *sn2* position [100, 101], we speculated that a possible determinant for the co-localization of FcγRIIIB and DiI-C_{16} may be the matching of the hydrophobic chain-length

between the respective lipid moieties of the two molecules. This hypothesis, previously proposed under the denomination of "mattress model" of lipid-protein interaction [120], could be tested by comparing the distributions of other derivatives of DiI with different methylene chain lengths, such as C_{12} and C_{18}, with that of aggregated FcγRIIIB.

Although I also present examples of DiI-C_{12} and DiI-C_{18} co-localization with aggregated FcγRIIIB in **Figure 22**, the success of these subsequent experiments was opposed by technical difficulties, relating to the fact that the variations in the length of the DiI methylene chains dramatically changed the diffusivity of these lipophilic probes and their partitioning to the cell membrane. Both these factors had a larger effect on the overall pattern of DiI labeling than that expected from hydrophobic chain-length matching alone.

DISCUSSION

The primary goal of this dissertation project was to determine the signaling capacity of FcγRIIIB, whose topology in the plasma membrane argues against its ability to function as a signaling receptor. The analysis of FcγRIIIB signaling is complicated by the fact that a second isoform, FcγRIIA, is also expressed on the neutrophil surface and shares the same ligand binding specificity as FcγRIIIB. Furthermore, the transmembrane isoform contains a cytosolic ITAM effector module with known signaling properties. In order to distinguish between signal activation by either GPI-anchored and transmembrane FcγR, isotype-specific mAbs were used to selectively engage either FcγRIIA or FcγRIIIB by direct antibody crosslinking. This allowed us to characterize the relative signaling phenotypes of the two receptor isoforms and ultimately propose a possible rationale for their co-existence.

The major findings of this investigation can be summarized as follows:

1. In the absence of direct ligand binding to FcγRIIA, and without pre- or co-stimulation by other exogenous factors, antibody crosslinked FcγRIIIB *is* capable of delivering an intracellular signal to the neutrophil, leading to robust $[Ca^{2+}]_i$ transients and receptor capping;

2. With few exceptions, the signaling phenotype of antibody crosslinked FcγRIIIB is identical to that of similarly crosslinked FcγRIIA, especially with regard to the susceptiblity of $[Ca^{2+}]_i$ transients to inhibition by wortmannin and N,N-dimethylsphingosine, which in turn inhibit PI 3-kinase and sphingosine kinase activity, respectively;

3. As visualized by immunofluorescence microscopy and by internalization assay, the physical disposition of both FcγR are remarkably similar under conditions of direct antibody crosslinking, both in the presence and absence of wortmannin. Furthermore, FcγRIIA co-localizes in the region of crosslinked and aggregated FcγRIIIB.

We therefore propose a model in which the segregation of FcγRIIA into lipid domains induced by crosslinking of GPI-anchored molecules, constitutes the initiation of the FcγRIIA signaling cascade and subsequent activation of the neutrophil immune response.

Signaling capacity of GPI-anchored FcγRIIIB

In light of the abundant evidence collected from studies of GPI-anchored protein activity in T lymphocytes, we were not surprised to observe that antibody crosslinked FcγRIIIB stimulates activity in the closely related neutrophil. However, this finding *does* have significance in the context of the longstanding controversy over the relative signaling roles played by FcγRIIIB and FcγRIIA. Clearly, the results of this investigation support the ability of the GPI-anchored isoform to function as competent receptor for intracellular signaling, corroborating previous reports that FcγRIIIB modulates $[Ca^{2+}]_i$ transients as well as neutrophil degranulation. [51, 121] Watson and coworkers recently reported that the mobilization of intracellular calcium by antibody crosslinked FcγRIIIB requires neutrophil "priming" with either tumor necrosis factor (TNF-α) or granulocyte-macrophage colony stimulating factor (GM-CSF). [122] However, I have not observed such a requirement for eliciting a response from crosslinked FcγRIIIB, and remain

uncertain of the reasons for this discrepancy, particularly since the methods that we used in our respective studies were very similar.

The dependence of the initial signaling event on Fcγ receptor crosslinking and aggregation appears to be even more critical for FcγRIIIB than for FcγRIIA. While we note that the inability of immobilized 3G8 Fab to stimulate frustrated phagocytosis in neutrophils was the only significant difference observed in the signaling properties of the two FcγR species, this seemingly anomalous result corroborates another interesting study which showed that, while T lymphocytes were activated by TCR/CD3 ligation to a cell-impermeant matrix, the same ligation of either GPI-anchored TAP or Thy-1 failed to have the same effect. [116] We believe that this phenomenon reflects the inherently different requirements for signaling by crosslinked FcγRIIIB, compared to FcγRIIA. While in principle, the activation of FcγRIIA by phosphorylation of ITAM can occur with the juxtaposition of just two receptors, the minimum number of GPI-anchored receptors required to form a lipid domain is presumably much larger. Clearly, the tethering of FcγRIIIB to immobilized GαM via 3G8 Fab would interfere with this clustering process. Our proposal that signaling by FcγRIIIB requires the formation of aggregates comprising a critical threshold number of receptors, is supported by other lines of evidence which show that bivalent ligands (and even low-valency immune complexes) are ineffective at triggering neutrophil activity through FcγRIIIB. [122, 123]

A technical issue which is relevant to the investigation of FcγRIIIB signaling is the choice of method used to specifically activate FcγR. While I have relied on isoform-specific 3G8 and IV.3 mAb to directly crosslink

FcγRIIIB and FcγRIIA respectively; other investigators have used Fab fragments of these same antibodies to selectively *exclude* FcγR from participating in crosslinking to nonspecific IgG complexes. Although the same objective of engaging a specific FcγR isoform is theoretically achieved, the results of experiments using the latter method generally attribute a much lowered signaling capability to FcγRIIIB than I have observed using the former technique. [124, 125] In light of our proposed model, I suspect that the addition of IV.3 Fab to block FcγRIIA binding to complexed IgG may in fact sterically oppose the close association of the transmembrane receptor with aggregated FcγRIIIB, which might otherwise occur if FcγRIIA were unbound. As a result, although the GPI-anchored receptor is indeed selectively crosslinked, the additional presence of IV.3 Fab may actually *inhibit* FcγRIIIB-mediated signaling. This suggestion, while entirely speculative, is supported by several previous studies in which neutrophil degranulation or $[Ca^{2+}]_i$ transients, triggered by complexed 3G8 mAb or other FcγRIIIB-specific target, was greatly attenuated by preincubation of PMNs with IV.3 Fab. [51, 126]

Pharmacologic inhibition of FcγR signaling

Implication of PI 3-kinase involvement

The involvement of PI 3-kinase in neutrophil FcγR signaling has already been extensively documented [127], and was in fact one of the first systems used to demonstrate the efficacy of the wortmannin inhibitor. [30] The purpose of using the PI 3-kinase inhibitor in this investigation, however, was to demonstrate the congruence of signaling by FcγRIIIB and FcγRIIA. The potent inhibition of FcγRIIIB-mediated calcium release by wortmannin suggests that PI 3-kinase is directly involved in its signaling pathway; yet the cytosolic enzyme, even when translocated to the inner leaflet of the plasma

membrane, cannot come in direct contact with the GPI-anchored receptor. Clearly, an intermediate signaling molecule is required to couple FcγRIIIB and PI 3-kinase, and I believe that FcγRIIA is the most likely candidate, since it not only co-localizes to the site of FcγRIIIB signaling, but inhibition of PI 3-kinase activity disrupts FcγRIIA signaling with identical efficacy.

The results from immunofluorescence microscopy and internalization assays, to track the movement of FcγRIIA and FcγRIIIB following antibody crosslinking, correspond exactly with those of a separate study showing that wortmannin does not block the internalization of PDGF or transferrin receptors, but dramatically inhibits the trafficking of endosomal vesicles to the lysosomal degradative pathway, and alters the structure and formation of endosomes through a yet uncharacterized mechanism. [128] These findings lend support to the possibility that the enzymatic activity of PI 3-kinase may also play a significant role in FcγR signaling. In the course of this investigation, polyphosphorylated inositol lipids, and in particular, those which are 3-OH phosphorylated, have gained increasing recognition for their functional involvement in regulating membrane traffic and endocytic transport processes. [129] For instance, PI (3,4,5)-trisphosphate, which is normally found in trace amounts in quiescent cells unless PI 3-kinase is activated, has been shown to function as a *bona fide* second messenger, activating *c-akt* (also called protein kinase B or PKB) by binding to its pleckstrin homology domain and recruiting an upstream kinase which phosphorylates *akt* on Thr^{308}, leading to activation of the proto-oncogene. [130]

Whether PI 3-kinase binds directly to phosphorylated FcγRIIA or indirectly via another phosphorylated intermediate remains to be determined. Nonreceptor protein tyrosine kinases of the *syk* family contain dual conserved SH2 motifs which can bind directly to various phosphorylated ITAMs. (see Isakov for review, [2]) Furthermore, crosslinking of FcγRII on promyelocytic HL60 cells has been shown to rapidly induce phosphorylation of $p72^{syk}$. [131] An interesting experiment to determine if PI 3-kinase is activated downstream of *syk* would be to examine the phosphorylation and activation state of PI 3-kinase upon FcγR stimulation in neutrophils preincubated with piceatannol, a specific inhibitor of *syk* kinase. [132] Nevertheless, we predict that the molecular basis for involvement of PI 3-kinase is the same for both FcγRIIA and FcγRIIIB-mediated signaling.

Evidence for an alternate intracellular Ca^{2+} release pathway involving sphingosine kinase

In light of a previous report by Rosales and Brown, which suggested a possible dissociation of FcγR-mediated $[Ca^{2+}]_i$ transients from the conventional IP3-dependent release mechanism [44], we demonstrate that N, N-dimethylsphingosine, a substrate analogue which competitively inhibits the production of sphingosine 1-phosphate by sphingosine kinase, inhibits the transient $[Ca^{2+}]_i$ rise mediated by ligation of FcγRIIA or FcγRIIIB, but not by stimulation of the fMLP receptor. Apart from confirming the convergence of the two FcγR signaling pathways, these findings support the existence of an alternate calcium mobilization pathway in the neutrophil, separate from the classic IP3 pathway. Sphingosine 1-phosphate was previously identified as the active signaling moiety in the autocrine stimulation of blood platelets [46] and was shown to trigger calcium-dependent proliferation of Swiss 3T3

fibroblasts. [133] Treatment of permeabilized fibroblasts with heparin to block IP3-gated calcium release, did not abrogate the calcium response to sphingosine 1-phosphate. [134] Kinet and coworkers demonstrated that sphingosine kinase mediates intracellular calcium release by an IP3-independent mechanism in rat mast cells (RBL-2H3), by showing that cells preincubated with the sphingosine analogue, D-L-*threo*-dihydrosphingosine, did not display intracellular calcium release upon stimulation by FcεRI, but were responsive to normal signaling by IP3. [47]

Although its exact mechanism of action is unknown, sphingosine 1-phosphate has been shown to mobilize calcium exclusively from thapsigargin-sensitive intracellular pools. [134] Pretreatment of neutrophils with thapsigargin was also used to help demonstrate that Fcγ receptor crosslinking and fMLP chemotactic peptide utilize two distinct pathways to mobilize calcium from a common cytosolic compartment [44] The mobilization of calcium from the same intracellular pool by two different second messengers and stimuli, sphingosine 1-phosphate by FcγR activation and IP3 by the fMLP receptor might seem counter-intuitive. However, we note that sphingosine 1-phosphate is probably membrane bound, as opposed to the water-soluble IP3. We therefore expect that signaling through FcγR-activated sphingosine kinase would produce a more localized calcium signal (appropriate for mediating phagocytosis, for example), than that triggered by the chemotactic peptide.

Functional role of FcγRIIA in signaling by FcγRIIIB

The striking similarity in signaling phenotypes of FcγRIIA and FcγRIIIB, as demonstrated by the evidence presented thus far, suggests that the

two receptors converge on a common signaling pathway at an early step following receptor activation. This functional link is validated by evidence from our immunofluorescence microscopy studies, which reveal that fluorescently labeled FcγRIIA co-localizes with crosslinked FcγRIIIB aggregates, in the notable absence of direct FcγRIIA ligation, suggesting that the two receptor isoforms are somehow physically associated on the plasma membrane as well. Since the end-distribution of FcγRIIA resulting from FcγRIIIB crosslinking is remarkably similar to the pattern observed when FcγRIIA itself is directly crosslinked, I propose that the transmembrane Fcγ receptors are sufficiently clustered under these conditions to initiate intracellular signaling by means of ITAM phosphorylation. (**Figure 20**) In this manner, I believe that the signaling phenotype of FcγRIIIB is determined largely by the characteristics and function of FcγRIIA. The requirement for a threshold number of FcγRIIA being brought into proximity within these cluster may contribute to the longer time delay observed for the mobilization of intracellular Ca^{2+} following FcγRIIIB crosslinking.

I attempted to validate this hypothesis using several different experimental approaches. From a functional standpoint, the dependence of FcγRIIIB signaling on FcγRIIA function could be demonstrated in neutrophils, under conditions where either the surface expression of FcγRIIA was downregulated, or FcγRIIA activity was competitively blocked by the incorporation of exogenous ITAM peptidomimetics. Unfortunately, neither of these experiments produced conclusive results. Prior antibody crosslinking of FcγRIIA in neutrophils, for example, failed to attenuate the subsequent calcium response triggered by FcγRIIIB crosslinking, probably because the transmembrane receptor was not entirely removed from the cell surface. It is

also conceivable that the biotin-IV.3 Fab-labeled FcγRIIA crosslinked by streptavidin somehow augmented signaling by FcγRIIIB, even though the FcγRIIA clusters presumably should have been sequestered from the cell surface.

As previously mentioned, the problem which most likely thwarted the successful inhibition of FcγR signaling by ITAM peptidomimetics was the method of incorporating the ITAM peptide into the neutrophil. While I was originally concerned that the neutrophils would no longer be viable after the long incubation with 10 mM peptide solution, the end-result was that the $[Ca^{2+}]_i$ transient elicited by FcγRIIA crosslinking was as robust as if the peptide (or its phosphorylated analogue) had not been present at all. We must consider that, for the incorporated ITAM peptide to have an effect, it ought to be localized at the inner plasma membrane surface, near endogenous FcγRIIA. Depending on the actual efficiency of loading, the cytosolic concentration of incorporated peptide may have reached levels as high as 100 μM. However, the effective concentration at the inner membrane surface may have been substantially lower, and insufficient to perturb signaling by FcγRIIA.

Physical model of GPI-anchored receptor function

The critical issue which remains to be addressed is the physical nature of the association between FcγRIIA and aggregated FcγRIIIB. Although in principle, any one of three modes of molecular interaction can occur, involving either the protein or the glycan or the lipid components of FcγRIIIB, several lines of evidence implicate the GPI anchor itself as the important signaling moiety. Apart from the collective studies on T

lymphocyte activation, which were previously described [88, 93, 96], GPI-anchored proteins other than FcγRIIIB (CD16) have also been shown to similarly trigger $[Ca^{2+}]_i$ transients in neutrophils, including CD48, CD55 (decay accelerating factor), CD58 (LFA-3), CD59, and CD67. [135] Finally, purified GPI anchors from *Plasmodium falciparum*, *Trypanosoma brucei*, and *Leishmania mexicana* were recently shown to function as agonists of cytokine production when added to macrophage host cells. [97] These results, in conjunction with our own experimental findings, argue against protein-protein interactions as the major determinant of FcγR co-localization. The other two modes of molecular interaction, involving primarily the GPI-anchor itself, have both been substantiated experimentally, and lead to two distinct hypotheses for signaling by GPI-anchored receptors.

Sehgal and coworkers presented intriguing evidence that FcγRIIIB-mediated $[Ca^{2+}]_i$ transients and superoxide production were greatly diminished in neutrophils exposed to 0.15M N-acetyl-D-glucosamine (NADG) or D-mannose, sugars which are common building blocks for the variable N- and O-linked chains which branch from the conserved core oligosaccharide chain covalently linking the protein to phosphatidylinositol [101] (ref. **Figure 2**), but not to the same concentration of other saccharides. [136] Exogenous NADG was also demonstrated to specifically disrupt co-capping of FcγRIIIB with complement receptor 3 (CR3), which is a beta 2 integrin (CD11b/CD18). [102] By noting that CR3 contains a putative lectin-like binding site which could recognize the glycosyl branches of GPI, Petty and colleagues proposed that the observed signaling by FcγRIIIB occurred by direct linkage with the integrin. [118] This model is appealing not only for its definition of a complete transmembrane signaling path for GPI-anchored

receptors, but also because integrins are known to play a variety of roles in modulating FcγR-mediated neutrophil activity. [137] However, several unresolved issues raised by this proposed mechanism lead us to question whether this truly represents the primary mode of FcγRIIIB signaling. In particular, an implicit stoichiometry is suggested by the direct coupling of CR3 molecules with a presumably random subpopulation of GPI-anchored proteins on the neutrophil surface. Assuming that the integrin is not differentially sensitive to aggregated vs. independent GPI-linked molecules, it is unclear how CR3 may selectively mediate activation by crosslinked FcγRIIIB. Most importantly, the studies by Petty and colleagues specifically exclude FcγRIIA from analysis, but do not address its possible role as a transmembrane signaling component of FcγRIIIB. While we believe that the natural proximity and signaling capacity of FcγRIIA makes it the ideal candidate for augmenting FcγRIIIB signaling, the demonstrated association of FcγRIIIB with other molecules such as the beta 2 integrin suggests the existence of other signaling partners (different from FcγRIIA) which could explain the observed synergism between the two FcγR isoforms.

Although the specific interactions which occur within the hydrophobic core of the membrane bilayer are among the least well-characterized, we believe that they form the basis for co-localization of FcγRIIA with aggregated FcγRIIIB. The physical evidence provided by previous studies of the colligative properties of GPI-anchored proteins, sphingolipids and cholesterol [76, 86, 87], together with the characterization of detergent insoluble membrane complexes involving GPI-anchored proteins and other signaling molecules [80, 83], led Simons and Ikonen to propose a formal model in which sphingolipids and cholesterol form functional "rafts" in the plasma

membrane, to which specific signaling molecules such as GPI-anchored proteins, transmembrane peptides, and acylated tyrosine kinases can dock and thereby perform integrated signaling functions across the membrane. (**Figure 23** and [103]) and We support the basic tenets of this model, but point out that FcγRIIIB appears to be uniformly and randomly distributed over the neutrophil membrane surface prior to antibody crosslinking. Therefore, we suggest that these rafts, if they indeed exist, may be transient entities which dynamically form and disassemble, driven in our specific instance by crosslinking of GPI-anchored molecules to multivalent ligand.

Clearly, one critical issue remains unresolved in this model: the nature of the cohesive force which attracts certain lipids, transmembrane proteins such as FcγRIIA, and other signaling molecules to form lipid domains with aggregated GPI-anchored proteins represented by FcγRIIIB. One hypothesis is that these components form thermodynamically stable, ordered microdomains enriched in phospholipids bearing saturated fatty acyl chains, as well as cholesterol and sphingolipids, which are thus resistant to low-temperature solubilization by nonionic detergents such as Triton X-100. [138] This notion was successfully tested by using placental alkaline phosphatase (PLAP), whose GPI-anchor contains dual saturated $C_{14:0}$ fatty acyl chains. However, it is not known whether this principle is equally valid for other GPI-anchored proteins with different lipid chains, such as those found in acetylcholinesterase and CD59, both isolated from human erythrocytes, which contain a highly unusual $C_{22:4}$ acylation at the glycerol *sn2* position, and an ether-linked $C_{18:0}$ at *sn1*. [100, 101] Since the chemical structure of the GPI-anchor for FcRIIIB is not yet known, it is unclear whether FcRIIIB also shares this acylation pattern, or if the pattern of lipidation in GPI-anchors is cell

lineage-specific. We postulated, however, that perhaps lipid molecules were organized around the GPI-anchored proteins on the basis of fatty acid chain length, since the packing of shorter chain phospholipids around GPI would be thermodynamically unfavorable due to the ensuing exposure of hydrophobic lipid surfaces to the aqueous environment. (**Figure 21**) The experiments which I attempted to perform in order to test this hypothesis, using various chain length DiI lipophilic probes, were not entirely successful for the technical reasons discussed above.

Although further investigations are clearly necessary to validate this proposed mechanism, the evidence I presented on the signaling capacity of FcγRIIIB, provides new insight into the functional architecture of the neutrophil plasma membrane. While the functional mechanism for many signaling molecules has been attributed to conformational or structural changes occurring within the molecule itself, the signaling capability of GPI-anchored proteins may rely on their ability to induce changes in the local composition of the intrinsically heterogeneous plasma membrane, thereby creating the conditions for the formation of supramolecular assemblies including all the components required for generating specific cellular responses upon contact with crosslinking immunogenic stimuli.

FIGURES & ILLUSTRATIONS

Figure 1. **Structural topology of various ITAM receptors.** Unlike T cell and B cell antigen receptors (as well as other Fc receptors), FcγRIIA contains both ligand binding and ITAM domains on a common transmembrane peptide chain. FcγRIIIB, on the other hand, contains only the extracellular IgG-Fc recognition domain, covalently linked to a GPI-anchor. (Schematic adapted from illustration in review by Isakov [2] and modified to include FcγRIIIB.)

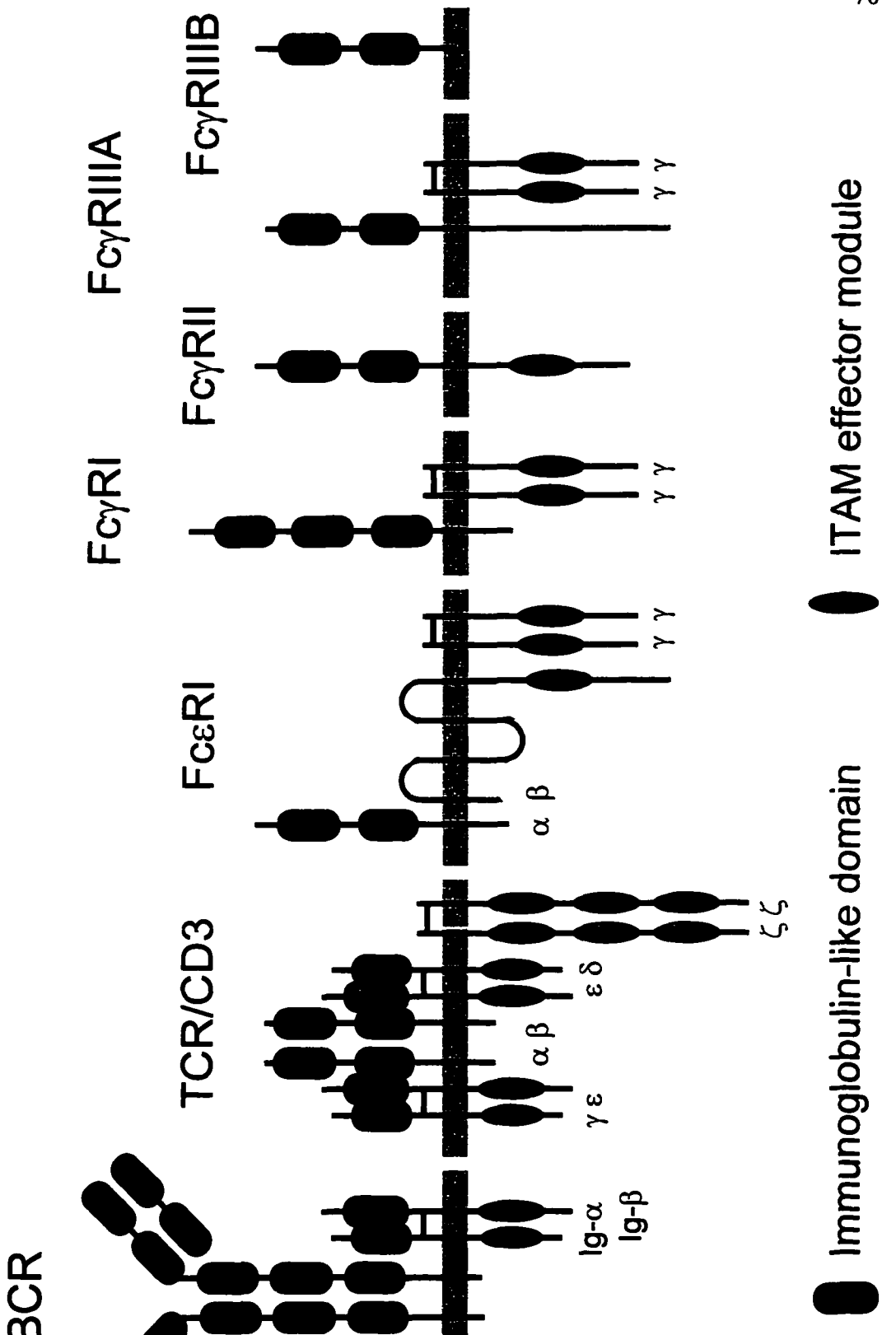


Figure 2. **Core structure of glycosylphosphatidylinositol anchor.** Despite the evolutionary diversity of GPI-anchored proteins, the structural arrangement of the core glycan chain linking ethanolamine to the inositol ring is well conserved. In several GPI-linked proteins (acetylcholine and CD59, for example, [100, 101]), the phosphatidylinositol moiety contains an unusual, multiply-unsaturated 22-carbon length chain at *sn*2, (R_2) as well as a separate palmitoylation of the inositol ring at the 3'-OH position. Arrows indicate hydrolytic sites of PI-specific phospholipase C and phospholipase D activity.

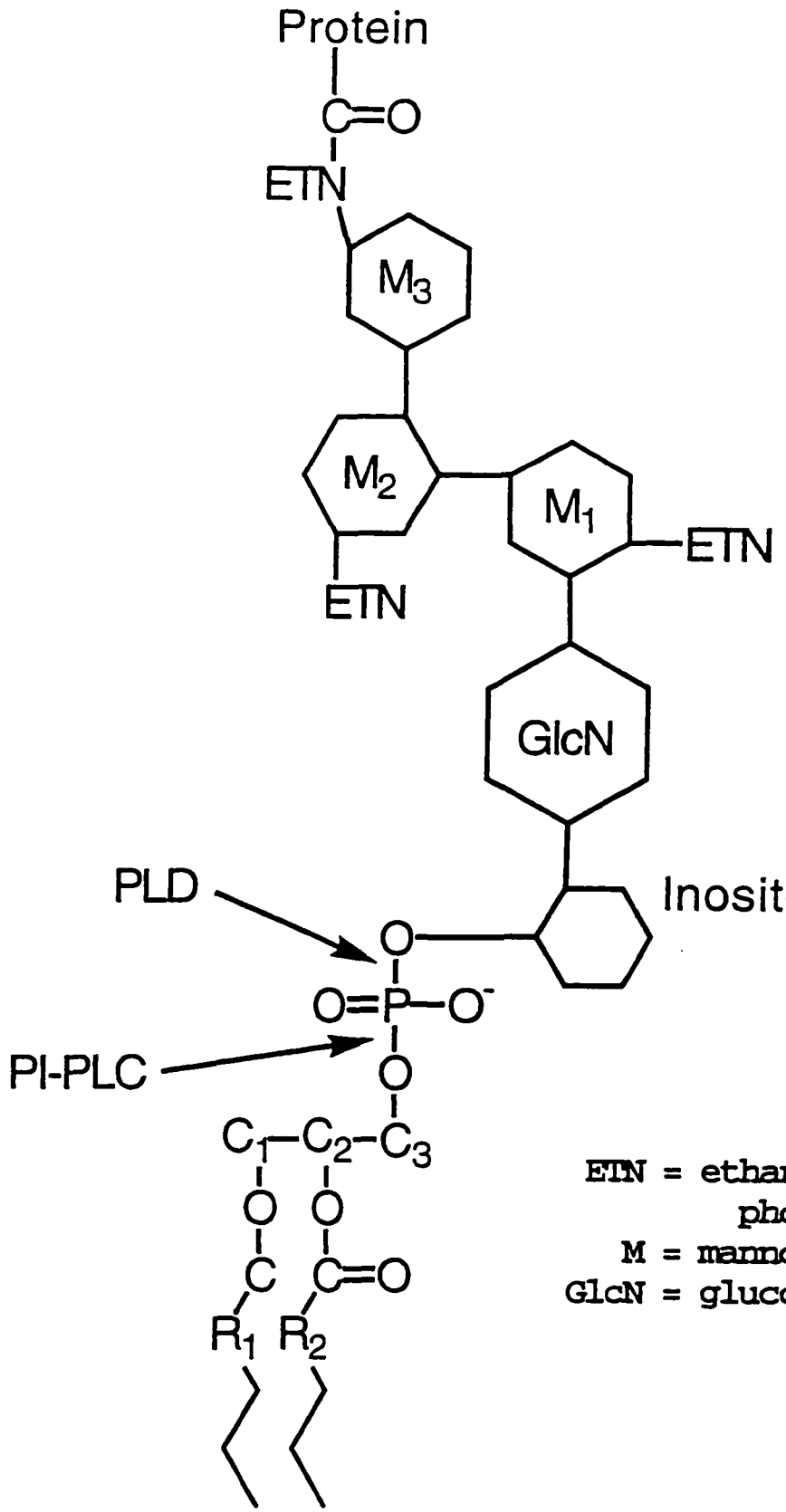


Figure 3. **Selective FcγR activation by direct antibody crosslinking.** Fab fragments of murine-derived 3G8 (anti-FcγRIIIB) or IV.3 (anti-FcγRIIA) mAb were used to specifically bind the FcgR species in question. Crosslinking was initiated by adding $F(ab')_2$ of polyclonal anti-mouse goat IgG (GαM).

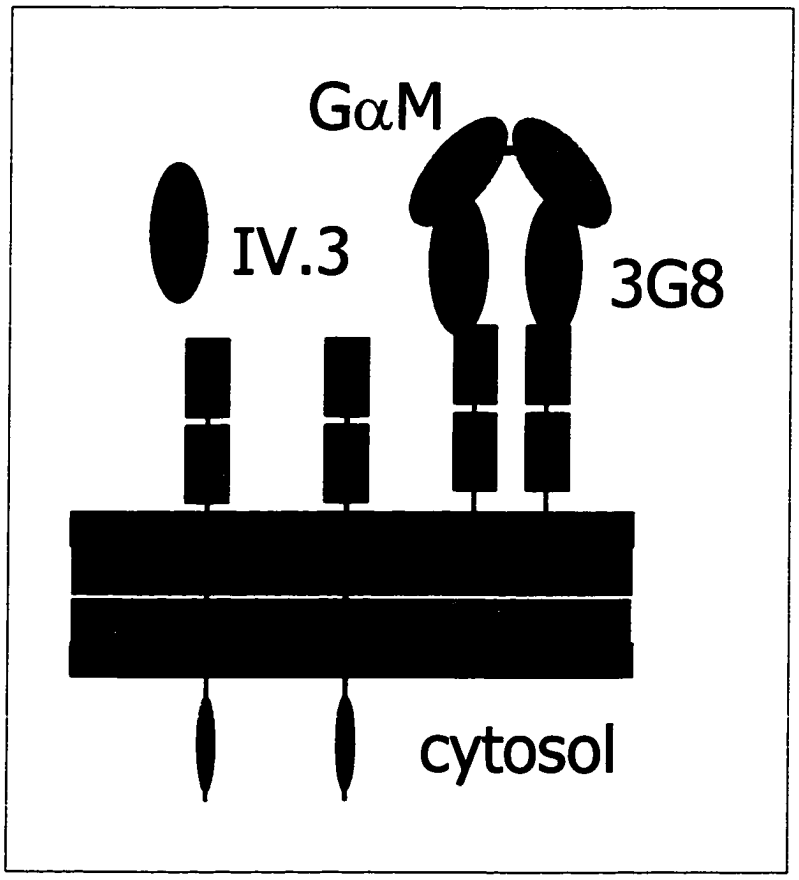


Figure 4. **Cell activation by "frustrated phagocytosis" assay.** PMNs or macrophages plated on derivatized glass coated with GαM, are triggered by the addition of IV.3 Fab, which tethers and crosslinks FcγRIIA onto the stationary surface.

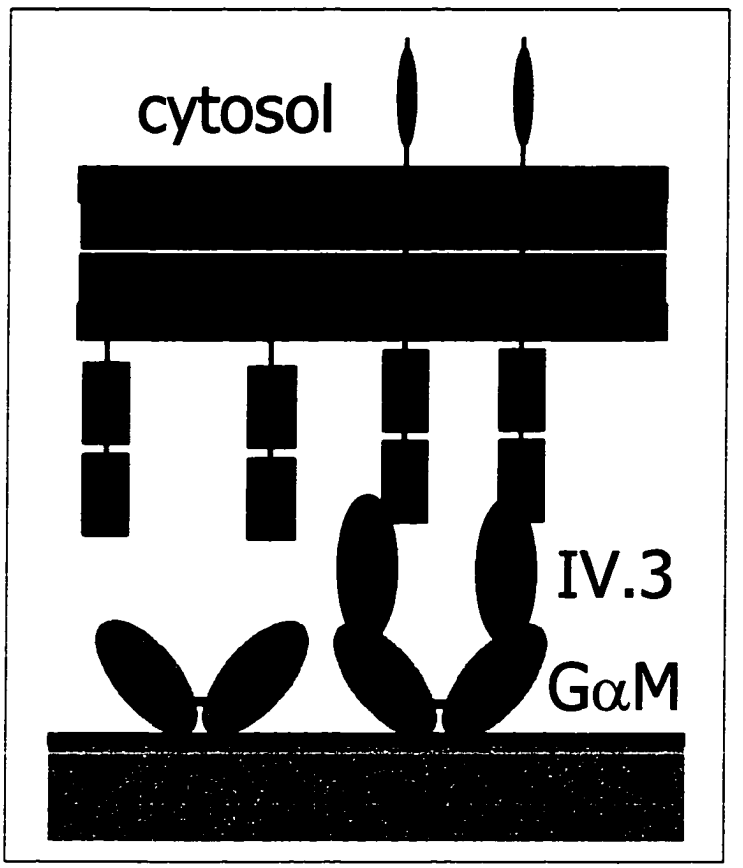


Figure 5. **The response to FcγR ligation of P388D1 macrophages, transfected with either wt or truncated FcγRIIA.** Cells were plated on coverslips coated with F(ab')2 anti-mouse IgG (GαM), and stimulated by adding 1μg/ml anti-FcγRIIA mAb IV.3 Fab. **A.** wt FcγRIIA, unstimulated control (NS); **B.** wt FcγRIIA, stimulated (S); **C.** Δ264, NS; **D.** Δ264, S; **E.** Δ233, NS; **F.** Δ233, S; **G.** wt FcγRIIA loaded with Ca^{2+} chelator BAPTA, S; **H.** wt FcγRIIA preincubated with genistein, S. (described in Methods)

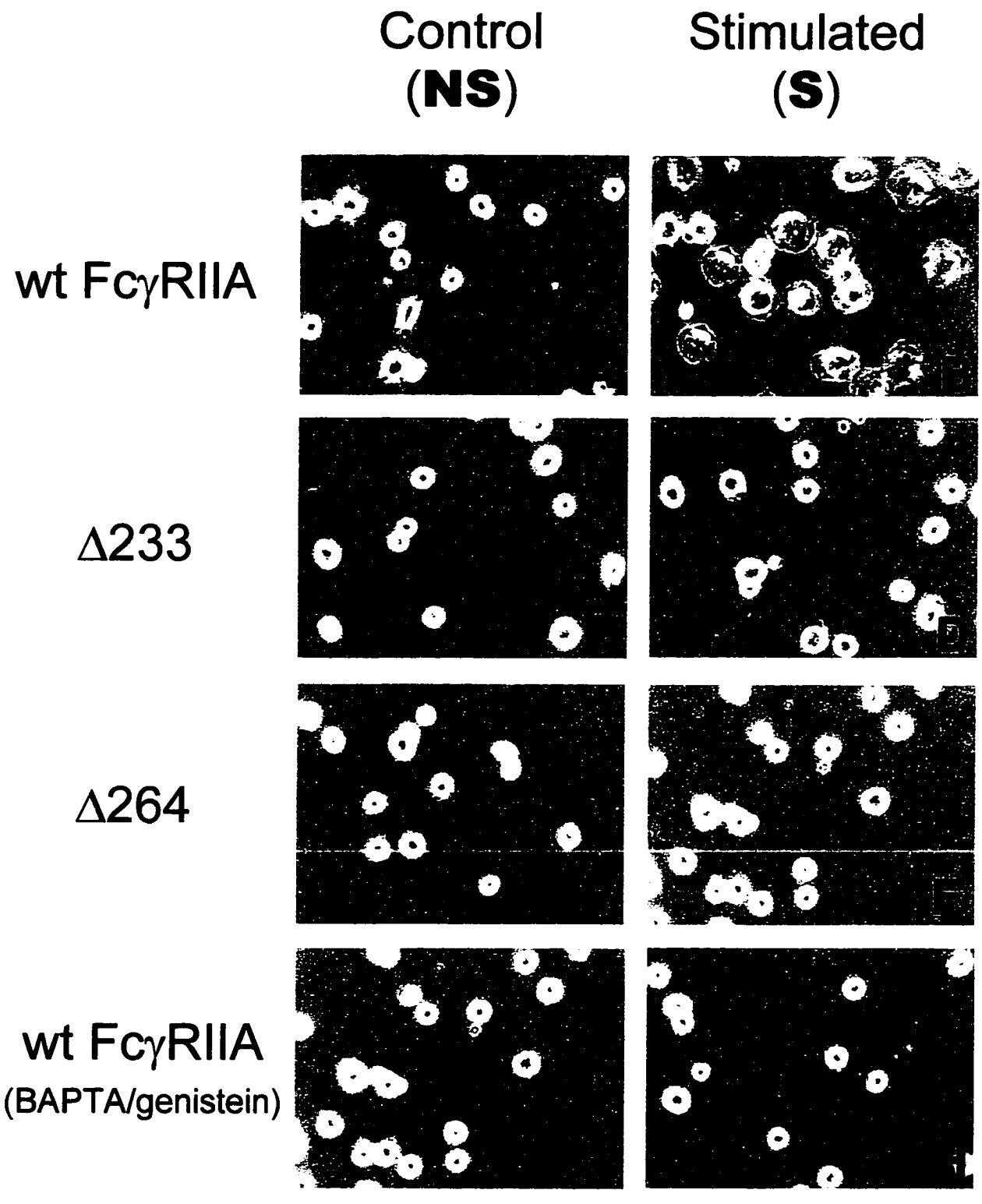


Figure 6. **Histograms showing the frequency distribution of the projected areas of cells expressing wt or mutated FcγRIIA during frustrated phagocytosis.** Data was collected on transfected P388D1 macrophages stimulated for 0, 10, or 60 min, using image-based cytometry (as described in Methods). While ligation of wt FcγRIIA evoked a rapid and sustained cell spreading response, no stimulation was observed with ligation of truncated FcγRIIA mutants Δ233 and Δ264.

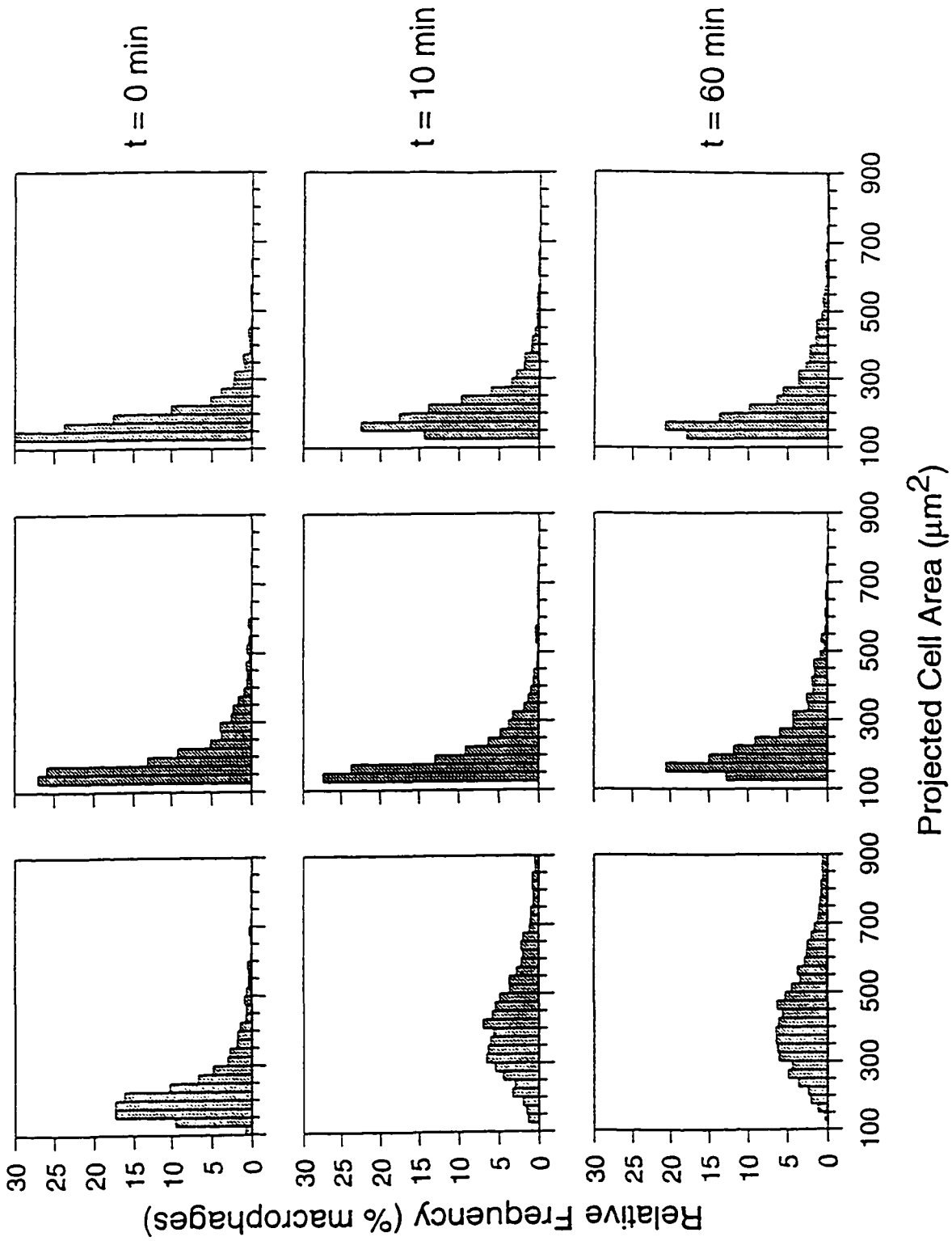


Figure 7. **Tethering of FcγRIIA (but not of FcγRIIIB), stimulates frustrated phagocytosis in human neutrophils.** Histograms represent the observed response of isolated PMNs plated on GαM-coated glass coverslips, 15 min after stimulation by the addition of either anti-FcγRIIA IV.3 or anti-FcγRIIIB 3G8 Fab. Unstimulated cells were used as a negative control.

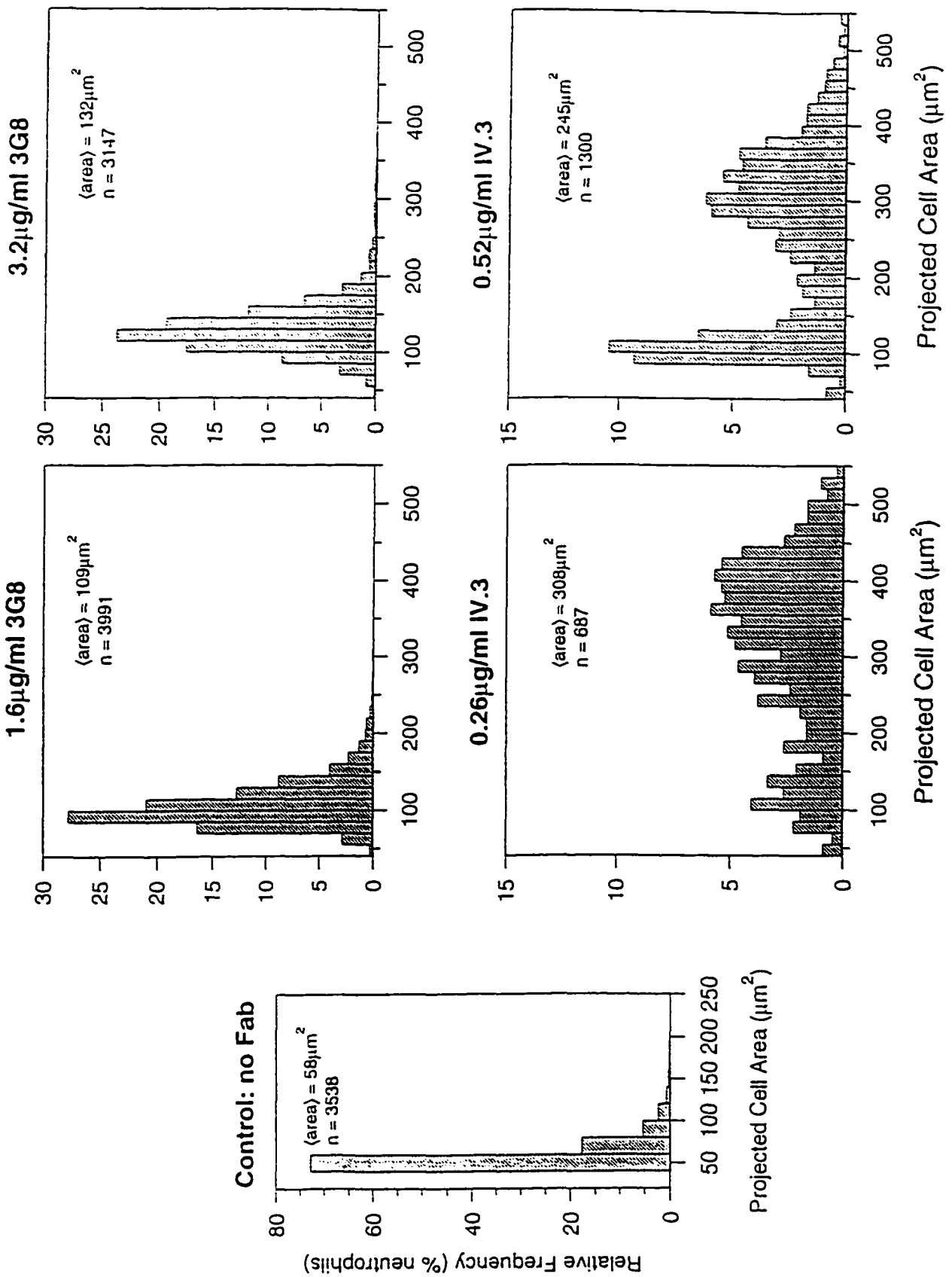


Figure 8. **Dose-dependent inhibition of FcγRIIA-mediated $[Ca^{2+}]_i$ transients and frustrated phagocytosis by the PI 3-kinase inhibitor, wortmannin.** Data acquired by Indo-1 calcium spectrofluorimetry (○,□) or by image-based cytometry of spread cells (●,■) of both neutrophils (○,●) and P388D1 cells transfected with wt FcγRIIA (□,■), plotted as percentage of maximum observed response (e.g., in the absence of wortmannin). Efficacy of PI 3-kinase inhibition by wortmannin was consistent for each cell type, with estimated $IC_{50} \approx 2$ nM and 23 nM in PMNs and macrophages, respectively.

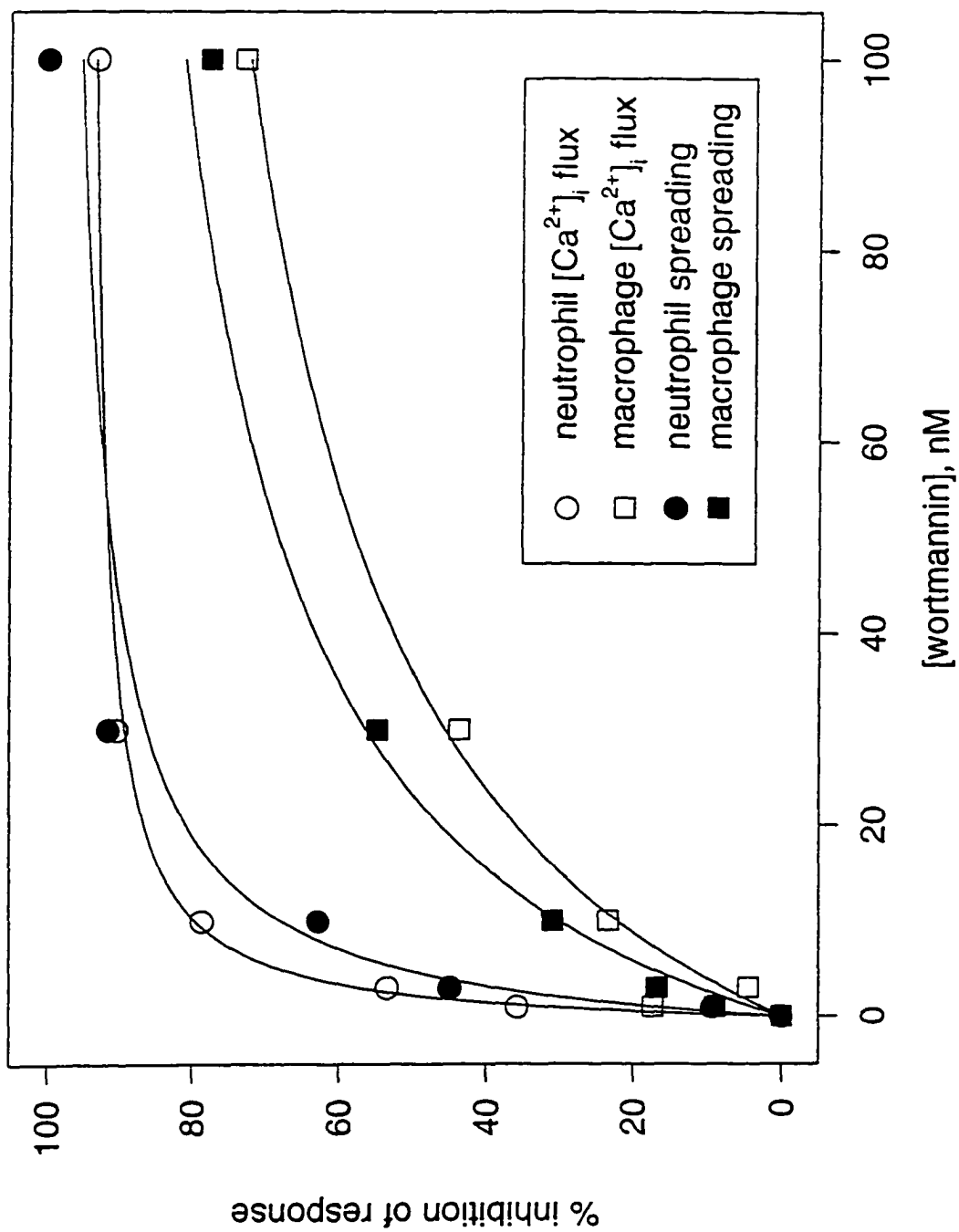
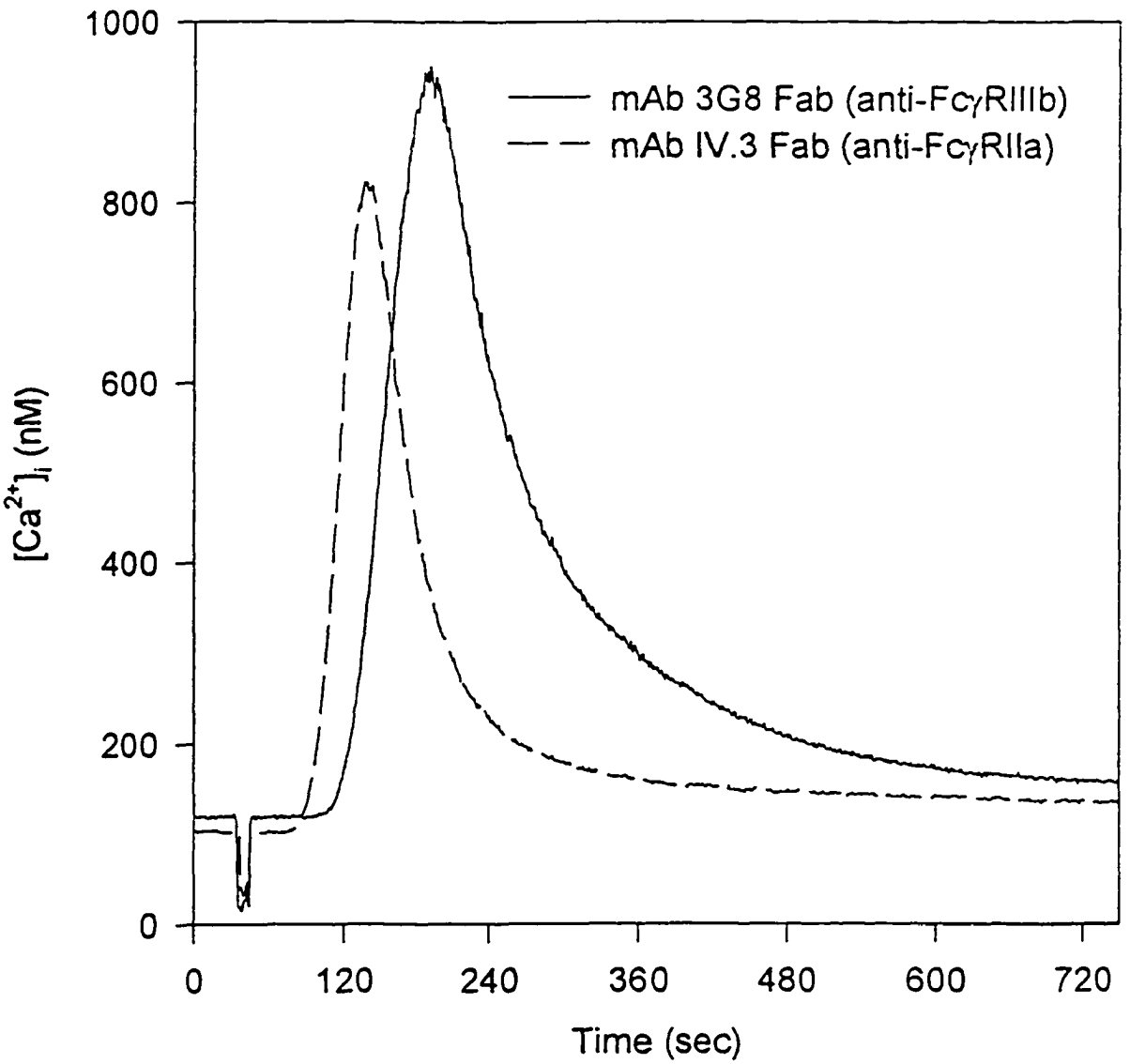


Figure 9. **Mobilization of intracellular calcium mediated by either FcγRIIA or FcγRIIIB, is equally susceptible to inhibition by wortmannin.**

Graph I Representative $[Ca^{2+}]_i$ transients in isolated human neutrophils, stimulated by direct GαM crosslinking of 3G8 Fab-labeled FcγRIIIB (solid lines) or IV.3 Fab-labeled FcγRIIA (dashed lines).

Graph II Dose response curves showing percent inhibition of peak $[Ca^{2+}]_i$ triggered by antibody crosslinking of FcγRIIIB (O), FcγRIIA (□), or fMLP stimulation (Δ).



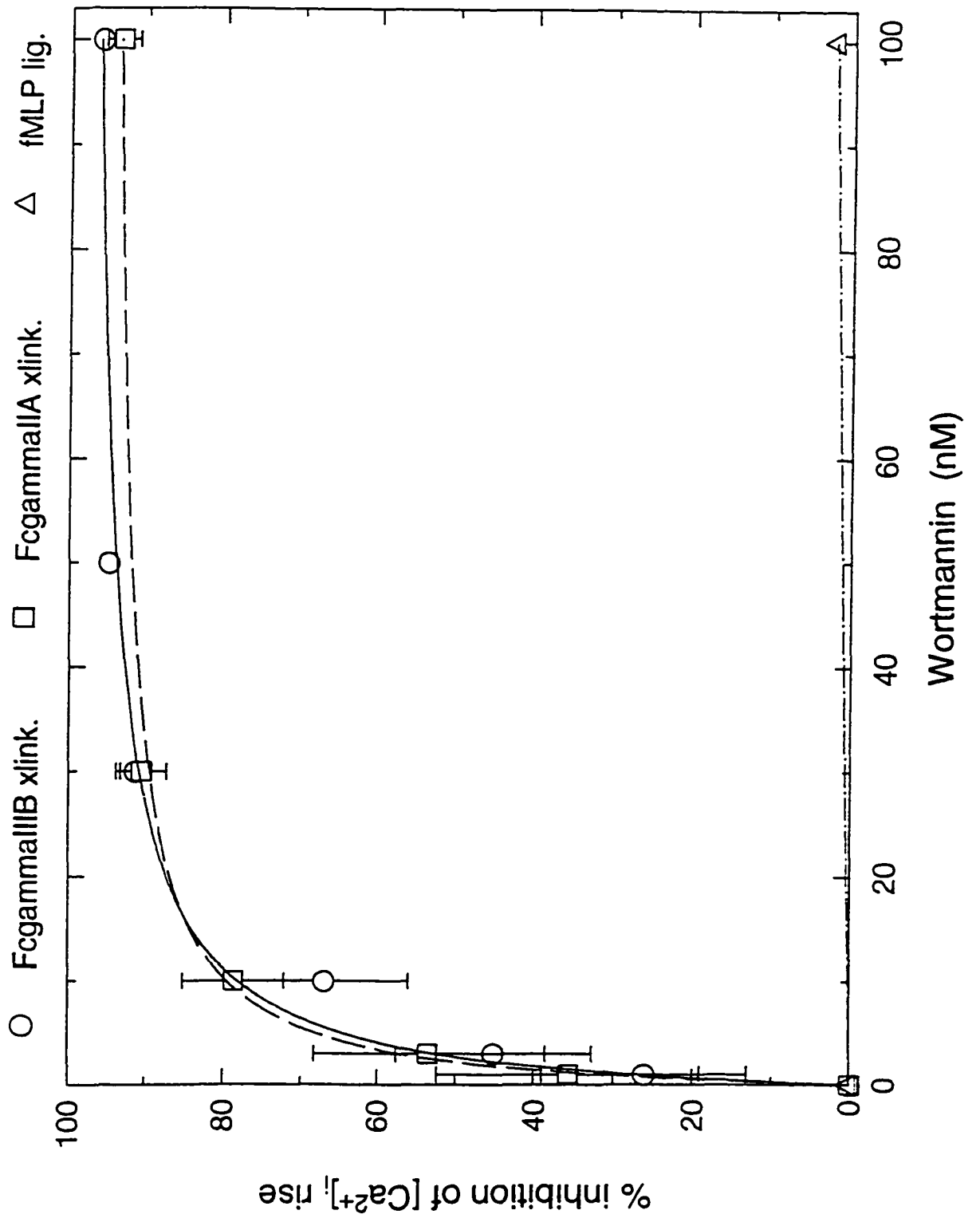


Figure 10. **Method for observing FcγR internalization.** ^{125}I-conjugated anti-goat F(ab')2 of rabbit IgG (RαG) is used to monitor the presence of crosslinking GαM on the neutrophil surface.

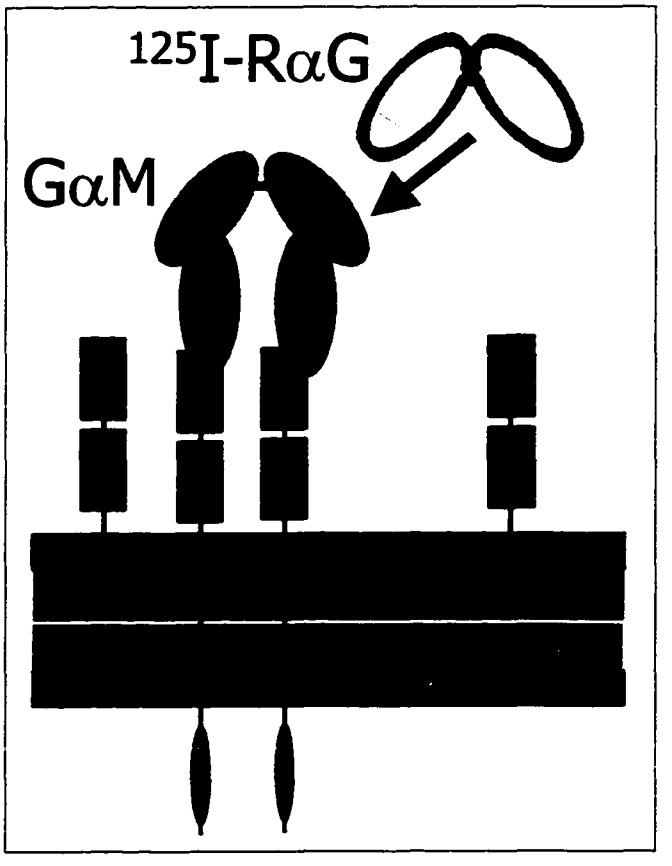


Figure 11. **Internalization of antibody-crosslinked FcγRIIIB or FcγRIIA is unaffected by PI 3-kinase inhibition.** Kinetics of sequestration of GαM-crosslinked 3G8 Fab-labeled FcγRIIIB (○,□) or IV.3 Fab-labeled FcγRIIA (●,■), in the presence (□,■) or absence (○,●) of wortmannin (100 nM), as determined by internalization assay. ^{125}I-conjugated F(ab')2 anti-goat IgG was used to probe for crosslinked FcγR on the cell surface. (described in Methods)

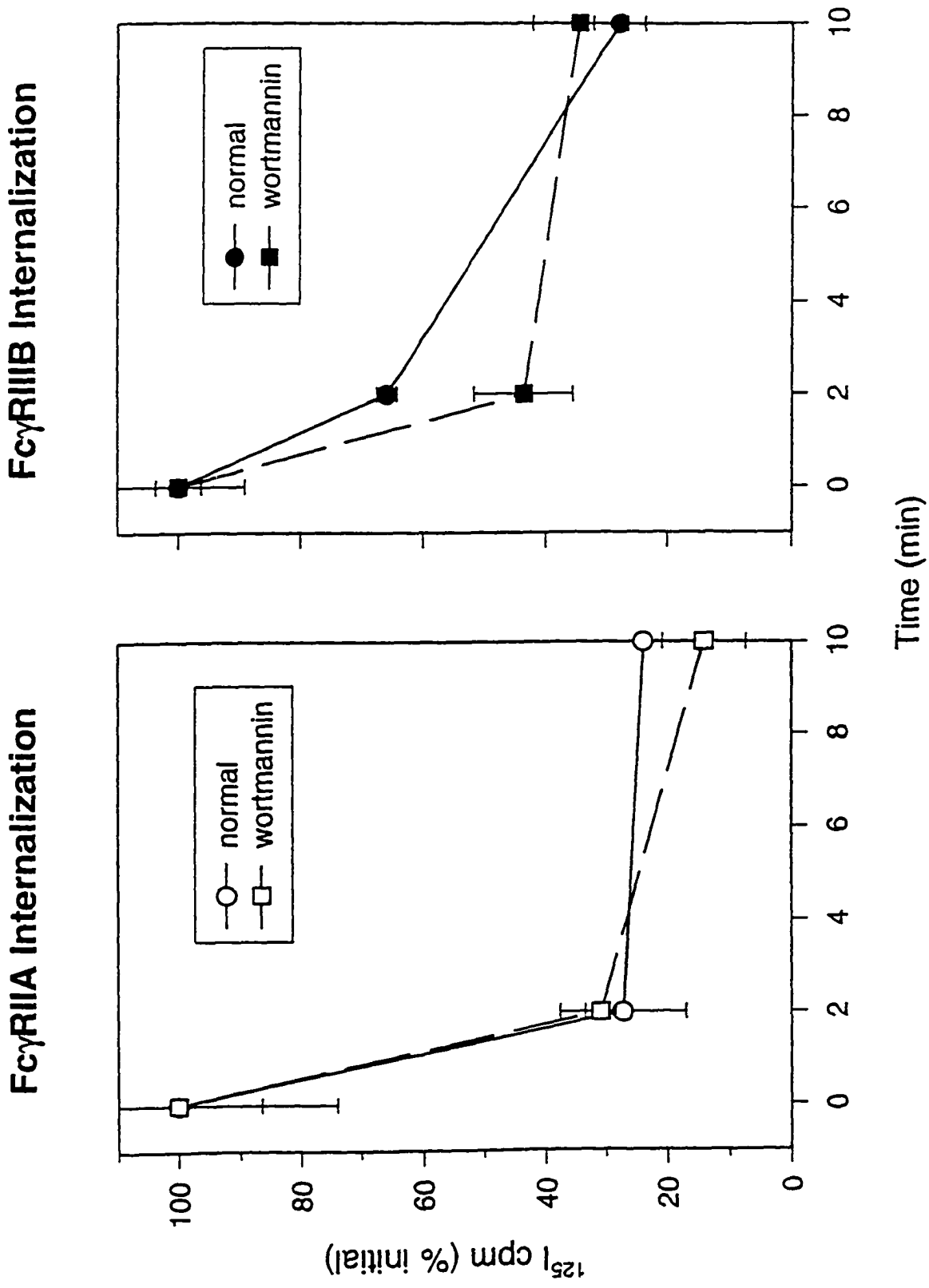
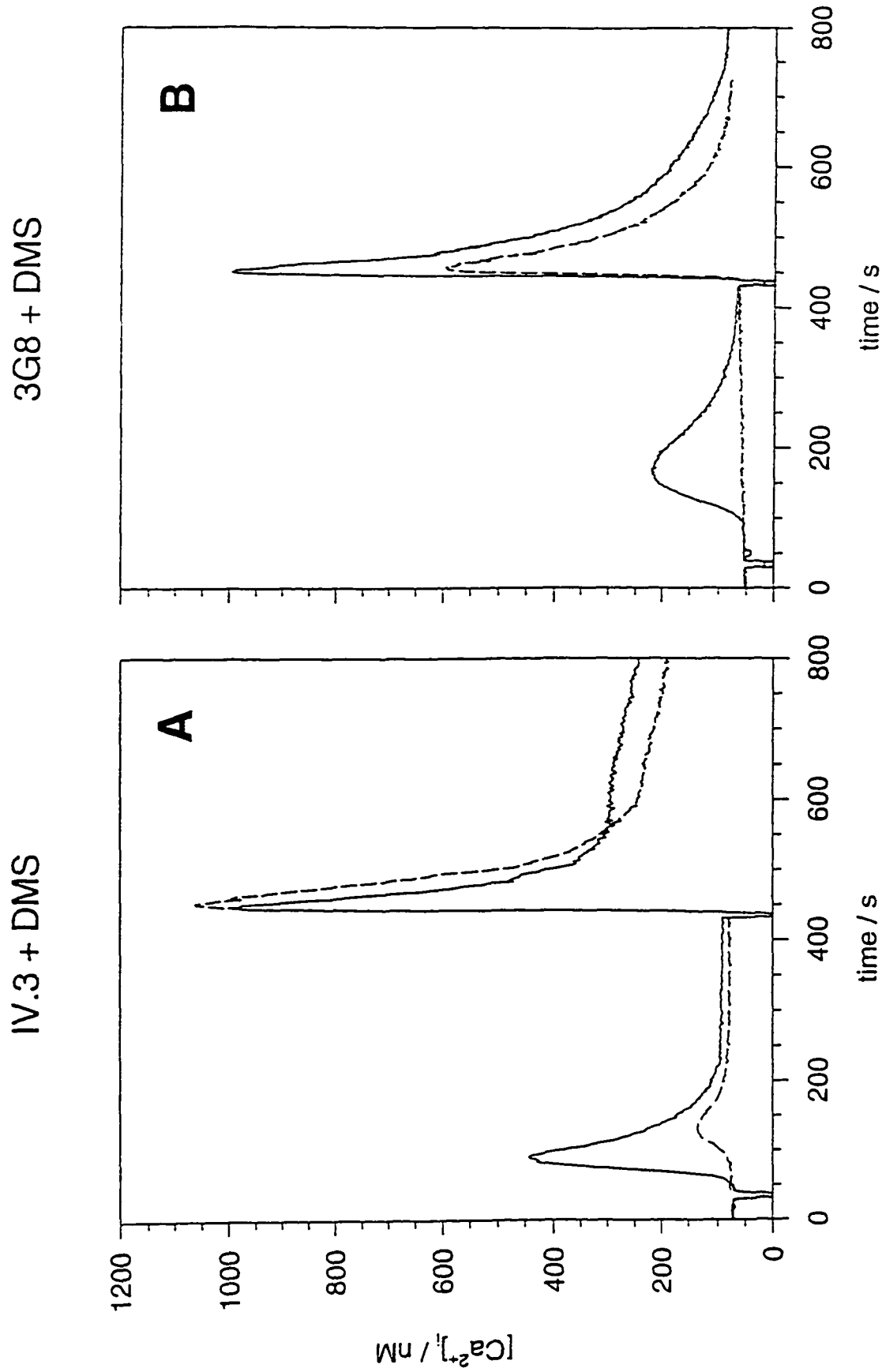


Figure 12. **N,N-dimethylsphingosine inhibits transient $[Ca^{2+}]_i$ rise stimulated by crosslinked FcγRIIIB or FcγRIIA, but not by fMLP.** Representative spectrofluorimetric $[Ca^{2+}]_i$ traces of Indo-1 loaded neutrophils, triggered by antibody crosslinked FcγRIIIB (plot **A**) or FcγRIIA (plot **B**), in the presence (dashed lines) or absence (solid lines) of N,N-dimethylsphingosine (~ 1 nanomole per 10^6 cells/ml), followed by 100 nM chemotactic peptide as a control stimulus; **C.** Summary of dose-dependent inhibition of FcγR-mediated calcium response by dimethylsphingosine.



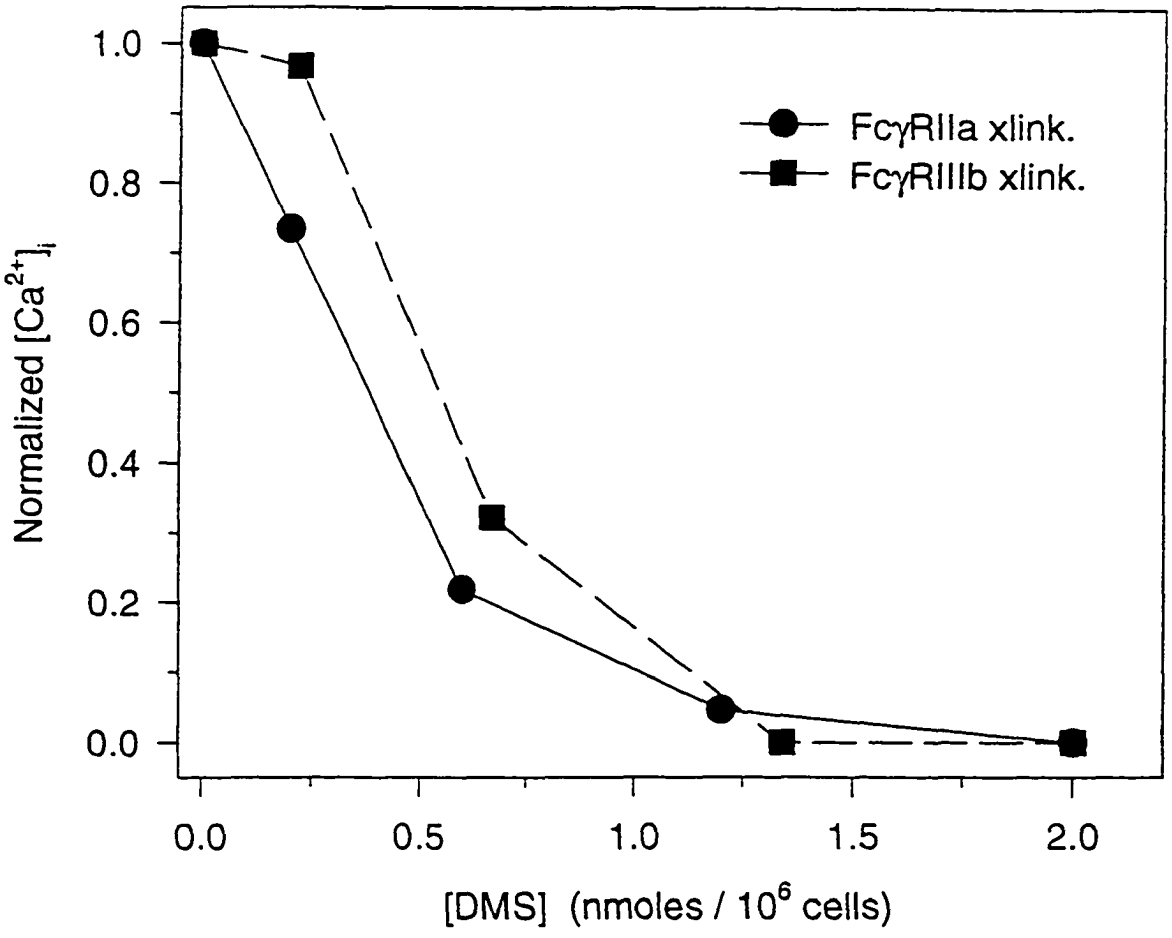


Figure 13. **Sequential stimulation of Fcγ receptor isoforms.** Representative $[Ca^{2+}]_i$ traces recorded from Indo-1 loaded neutrophils labeled with both biotinylated IV.3 Fab and unconjugated 3G8 Fab. **A.** FcγRIIA was first crosslinked with streptavidin (30 μg/ml) to induce receptor internalization. FcγRIIIB was subsequently crosslinked with GαM (30 μg/ml), followed finally with stimulation by chemotactic peptide (100 nM); **B.** Same procedure, on cells labeled only with 3G8 Fab; **C.** Same procedure, on cells labeled only with biotin-IV.3 Fab.

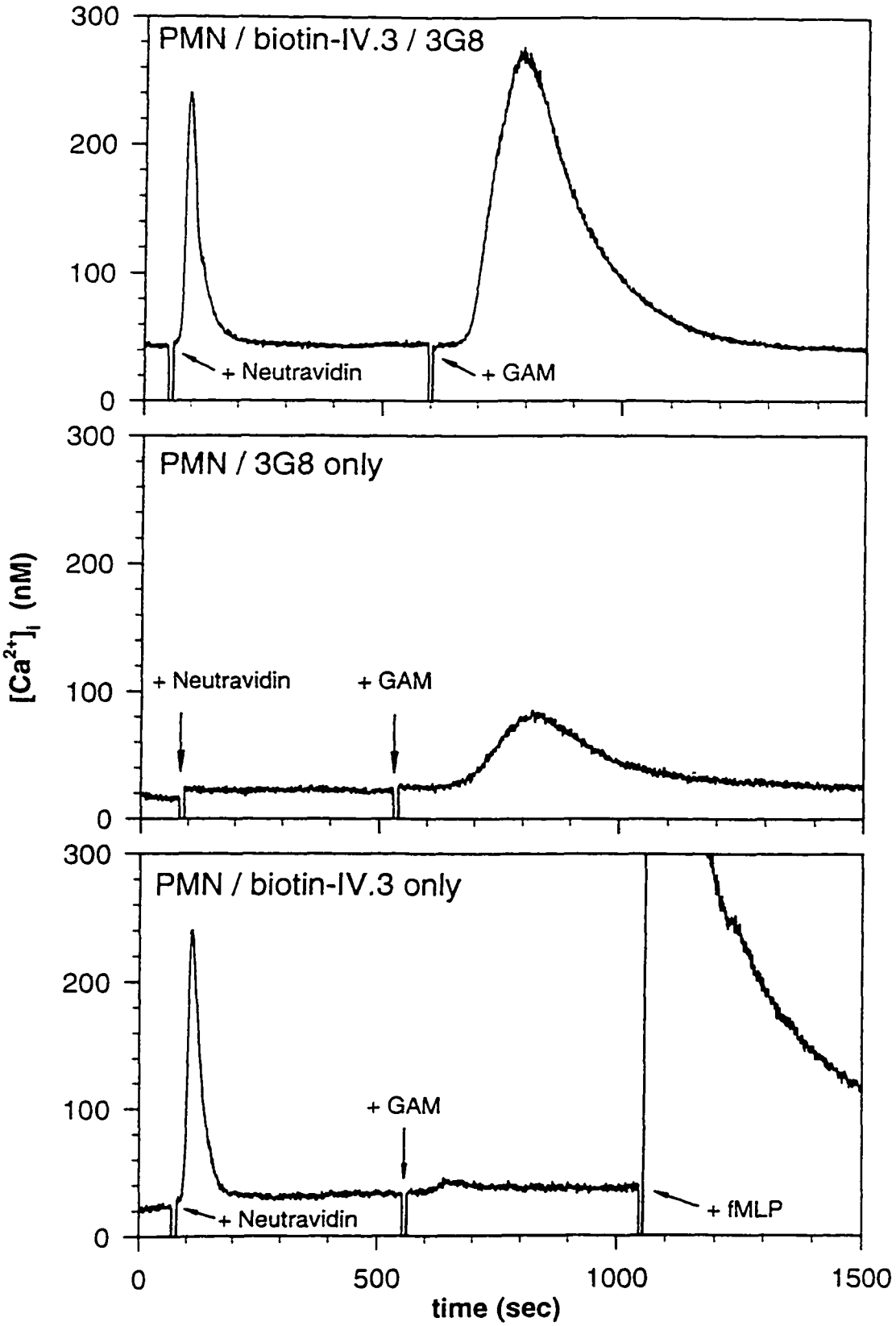
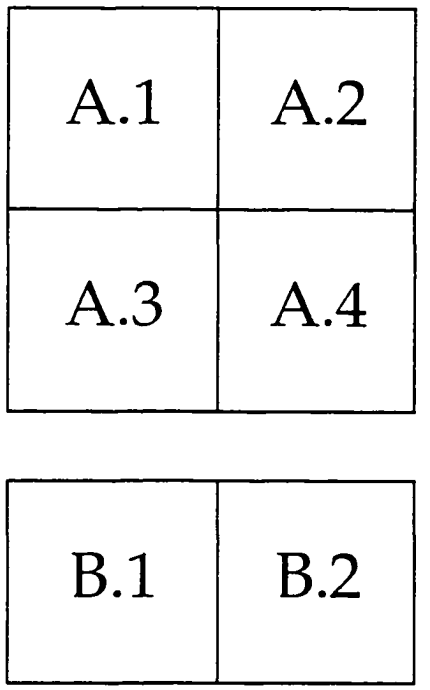
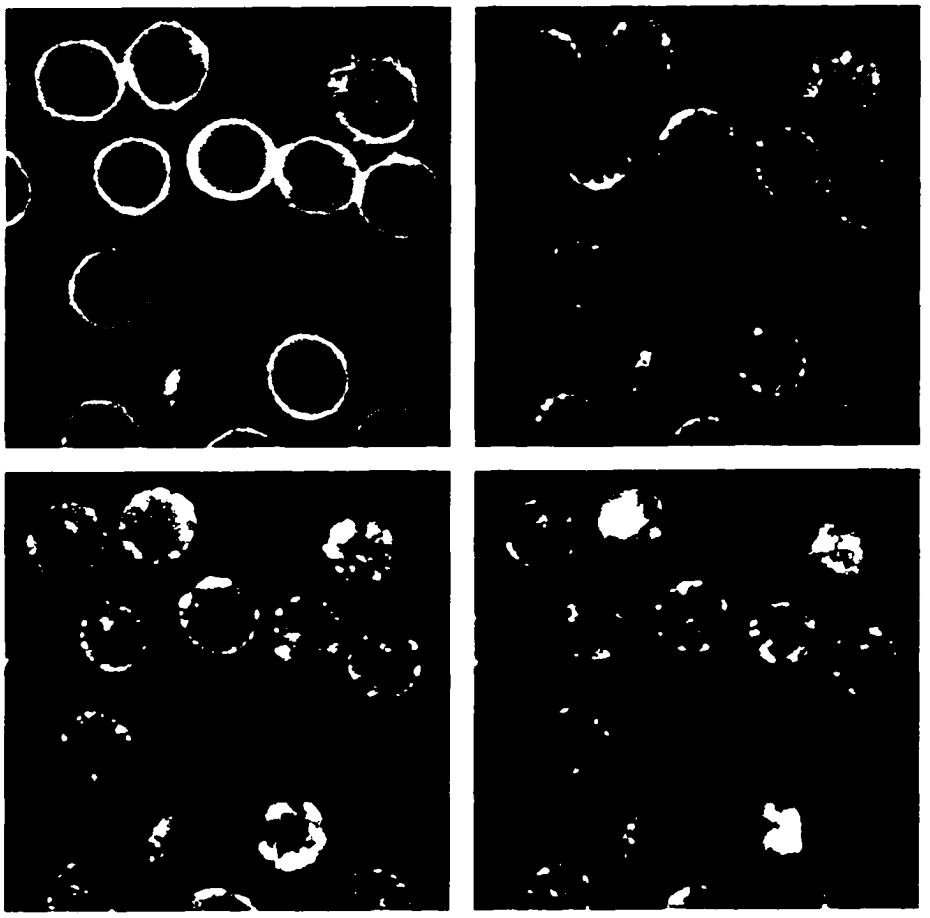


Figure 14. **Aggregation of FcγRIIIB following direct antibody crosslinking.**



A.1-4. Image sequence showing the formation of fluorescein-3G8 Fab labeled FcγRIIIB receptor caps, which occurs within 7 min of crosslinking with GαM at rt. Analagous results were obtained with antibody crosslinked FcγRIIA (not shown); **B.1-2.** On rat basophilic leukemia cells (RBL-6CD5) transfected with huFcγRIIIB, the distribution of labeled receptor is initially patched, and does not coalesce into larger aggregates upon receptor crosslinking.

Neutrophils



RBL-6CD5

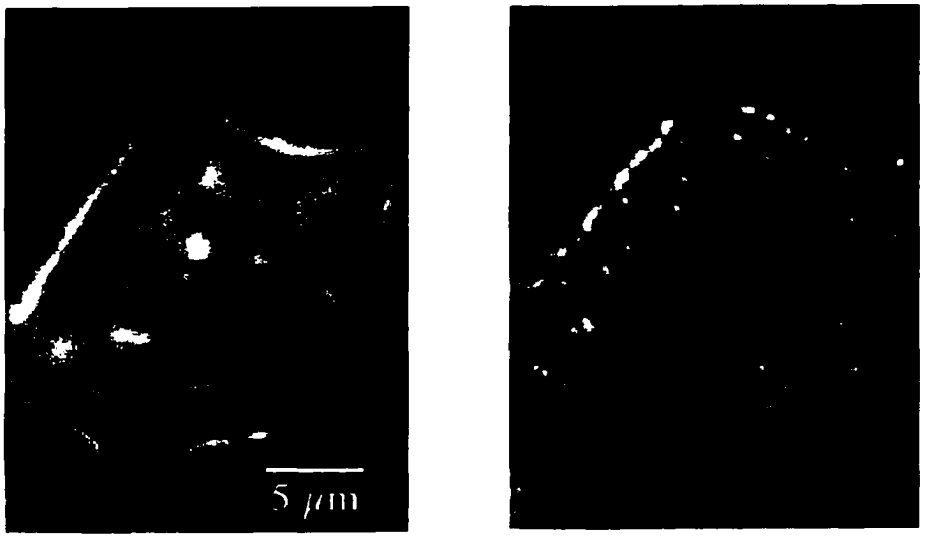


Figure 15. **Wortmannin disrupts pattern of FcγR aggregation.** Fluorescence confocal images of neutrophils labeled either with rhodamine-conjugated 3G8 Fab (**left panels**) or with fluorescein-conjugated IV.3 Fab (**right panels**), approximately 7 min after crosslinking with 30 μg/ml GαM. (described in Methods) In neutrophils preincubated with wortmannin (30 nM, **lower panels**), the FcγR aggregates are dispersed throughout the cytosol, compared to the receptor capping behavior normally observed. (**upper panels**)

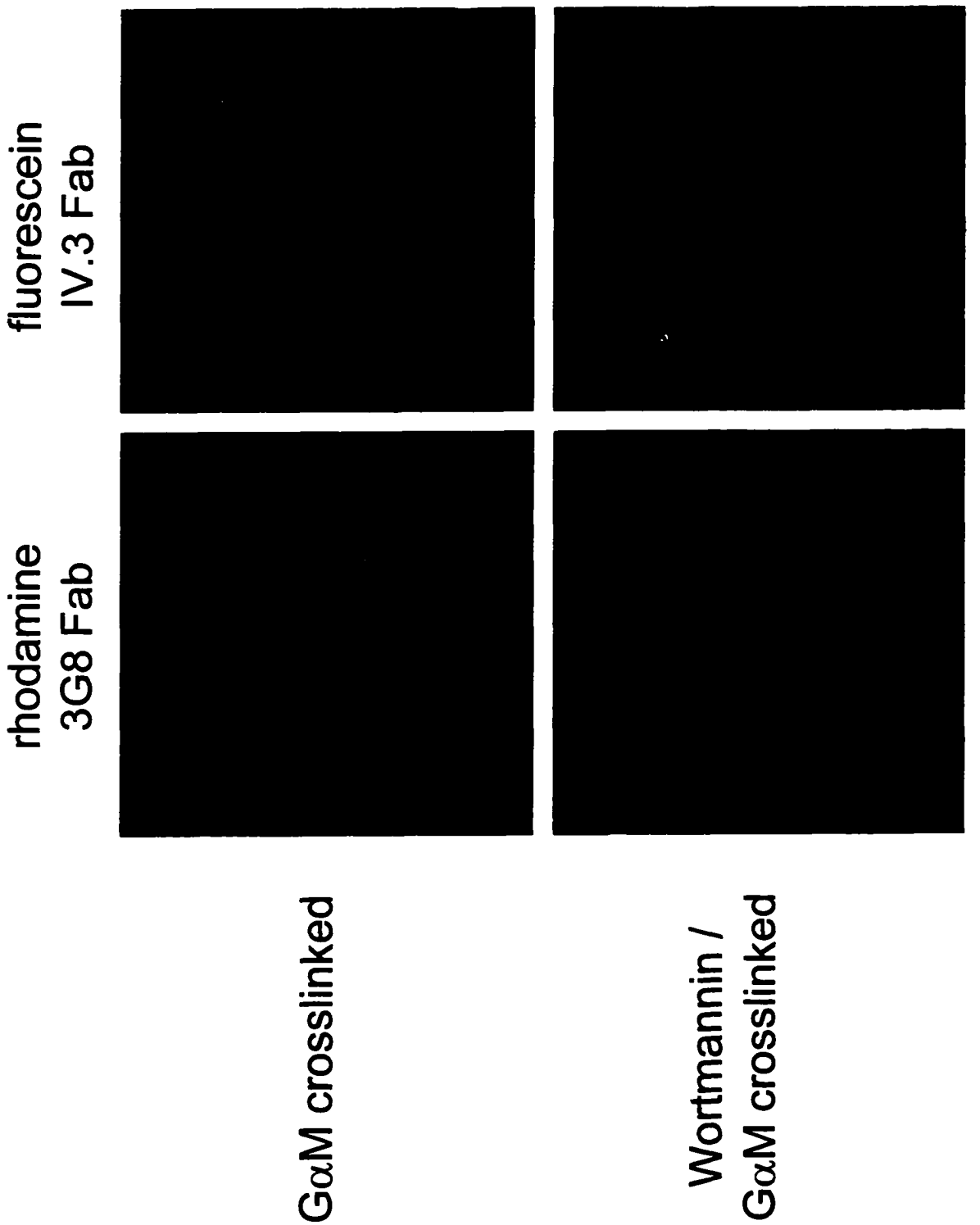
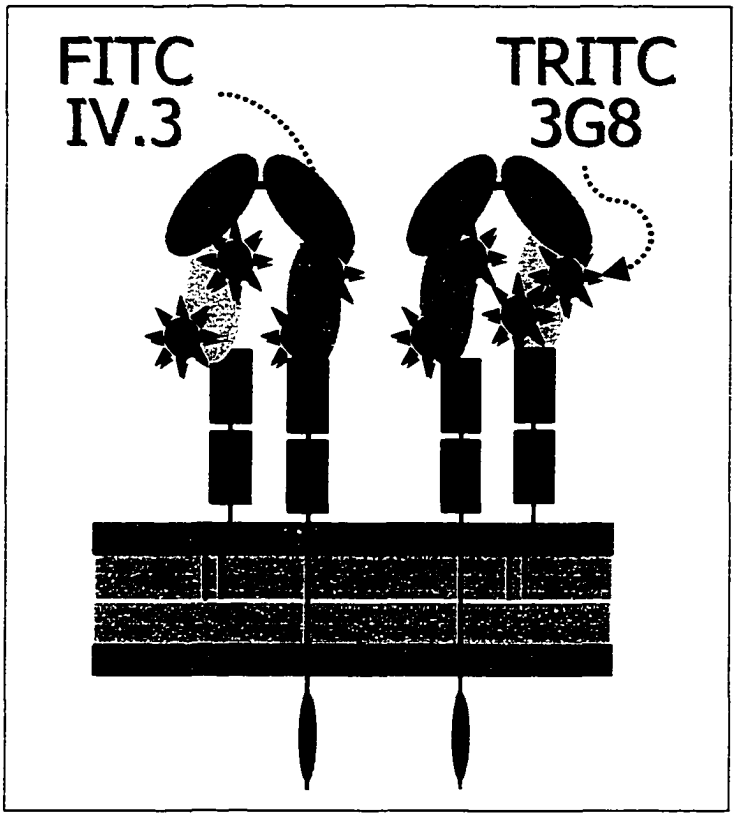


Figure 16. **Fluorescent double-labeling strategies to visualize FcγR co-localization.** To exclude co-localization by heterotypic crosslinking, 3G8 Fab were derivatized with long-chain (LC) biotin, and crosslinked with Texas Red streptavidin.



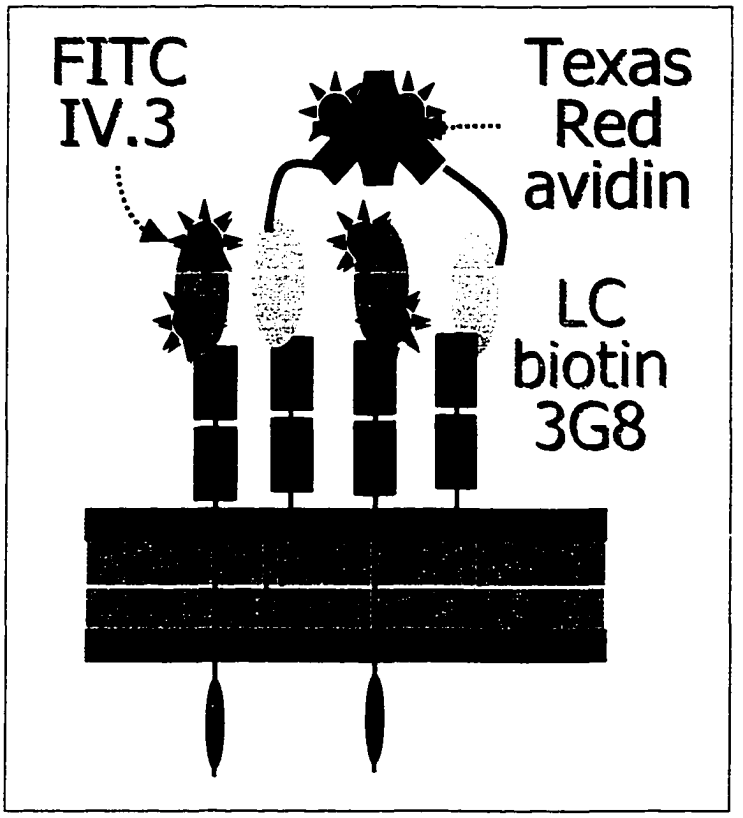
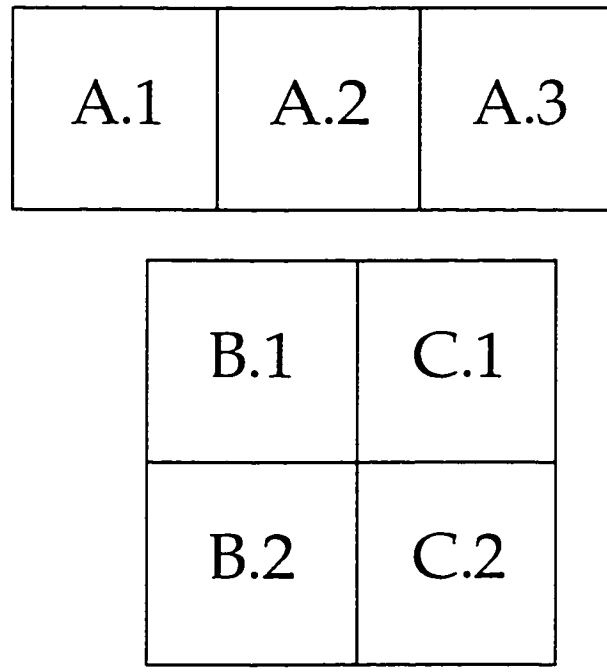


Figure 17. **FcγRIIA co-localizes with antibody crosslinked FcγRIIIB.**



A.1-3 Conventional fluorescence photomicrographs showing overlapping Fcγ receptor distributions. Texas Red-conjugated streptavidin (15 μg/ml) was added to neutrophils previously labeled with both fluorescein-IV.3 Fab and biotinylated 3G8 Fab. After crosslinking 7 min, the cells were cytospun onto glass and fixed in cold methanol. (as described in Methods); **B.1-2.** Confocal images of similarly prepared cells; **C.1-2.** Heterotypic crosslinking of rhodamine-3G8 Fab labeled FcγRIIIB with fluorescein-IV.3 Fab labeled FcγRIIA, using 30 μg/ml GαM.

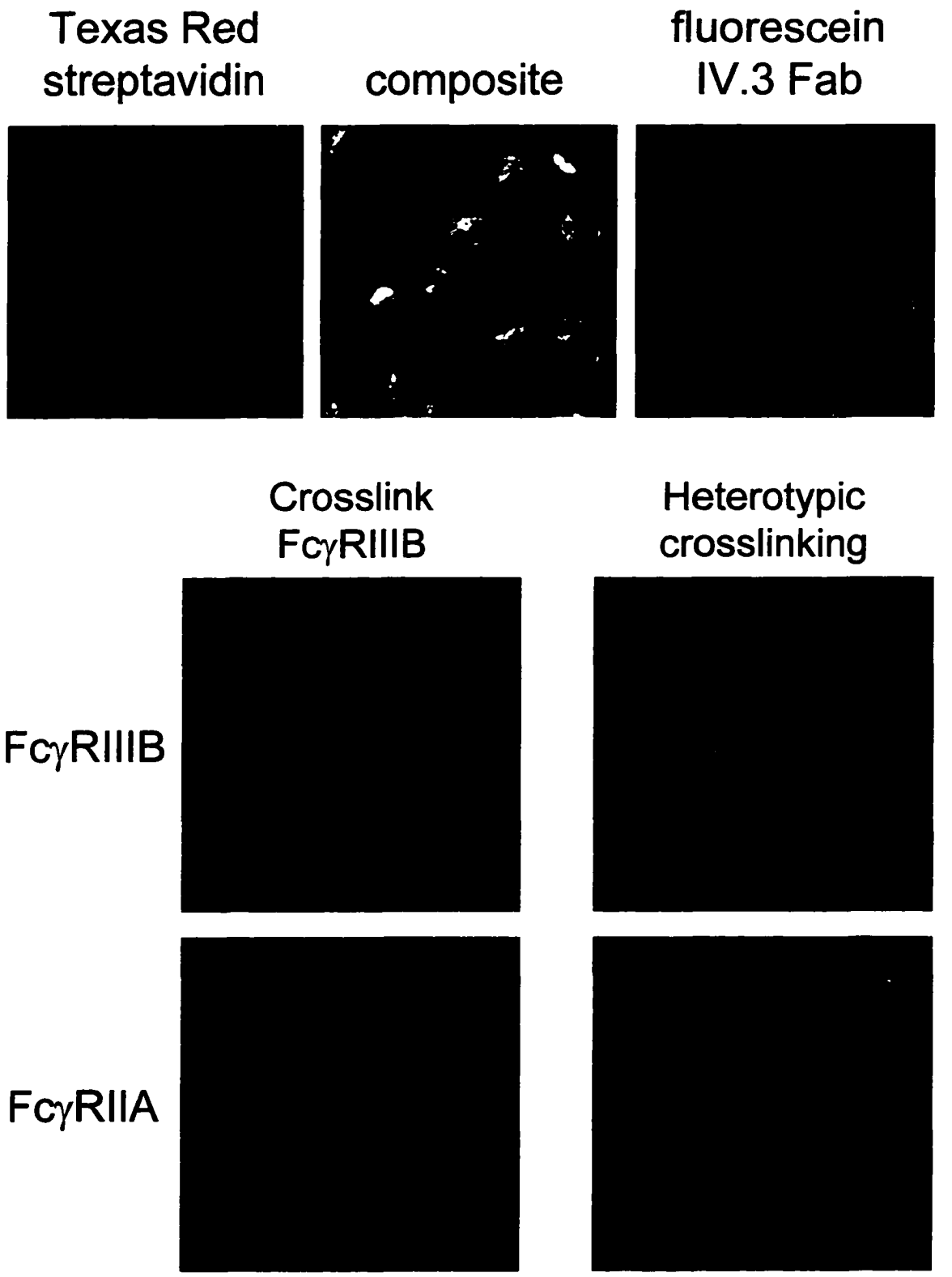


Figure 18. **Experimental setup for time-resolved fluorescence spectroscopy using far-field optics.** Schematic design by Dr. Jay Trautman (formerly of Bell Laboratories, Murray Hill, NJ).

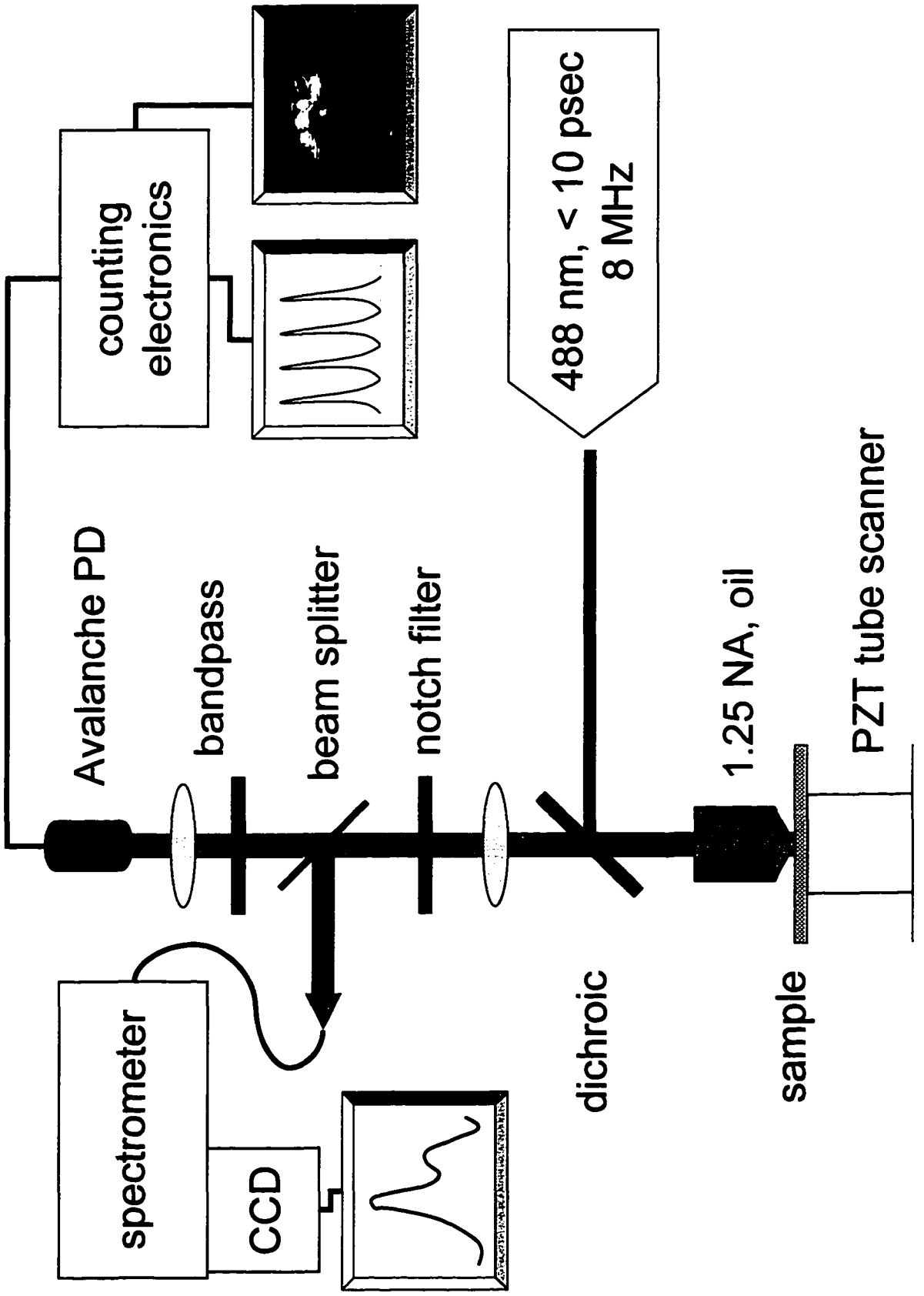
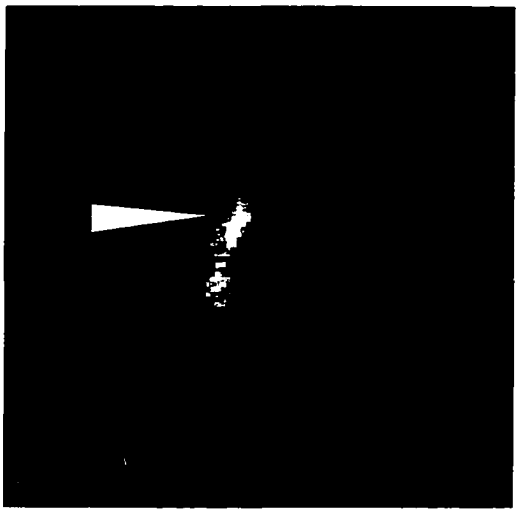
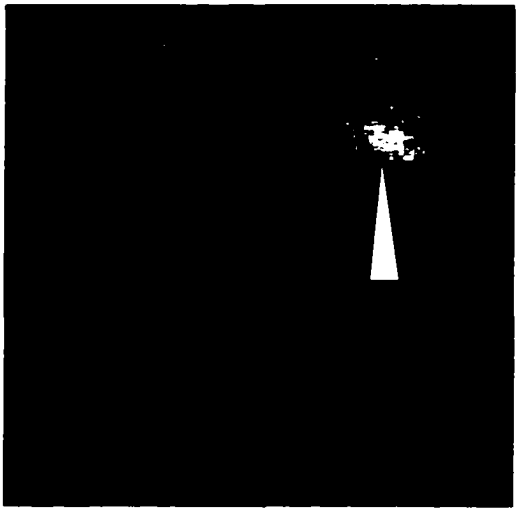


Figure 19. **Summary of fluorescence resonance energy transfer (RET) analysis of co-localized FcγR.** Fluorescent-labeled Fab probes located within FcγR clusters were analyzed for RET, using time-resolved spectroscopy. Neutrophil preparation and experimental setup are described in Methods (also in the Appendix). Basically, after an initial scan to select a region for analysis (**first set**, arrows indicate selected target areas), the fluorescence emission from selected cluster analyzed spectrally (**second set**) as well as by fluorescence excitation decay (**third set**). The results of three separate trials are shown: **A**. FcγR cluster containing both fluorescein IV.3 and rhodamine 3G8 Fab; **B**. FcγR cluster containing fluorescein IV.3 only; **C**. FcγR cluster containing only rhodamine 3G8 (excited at rhodamine wavelength). The similarity in fluorescein lifetime measurements in **A**, relative to **B**, indicates that RET did not occur between fluorophores in sample **A**.

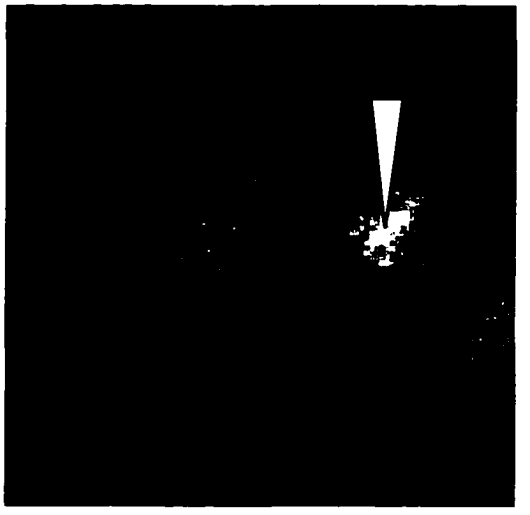
A. FITC-IV.3 / TRITC-3G8

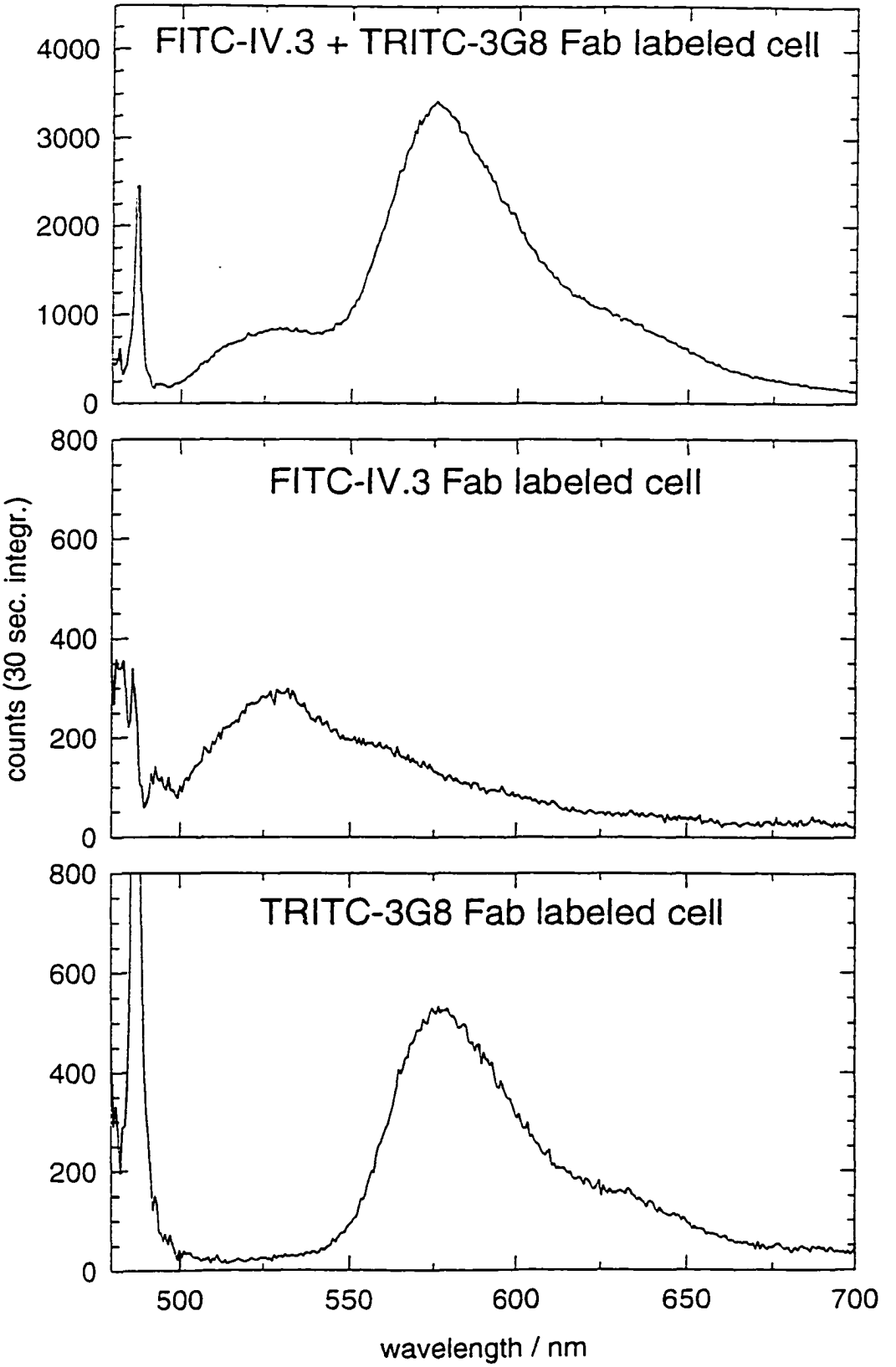


B. FITC-IV.3 only



C. TRITC-3G8 only





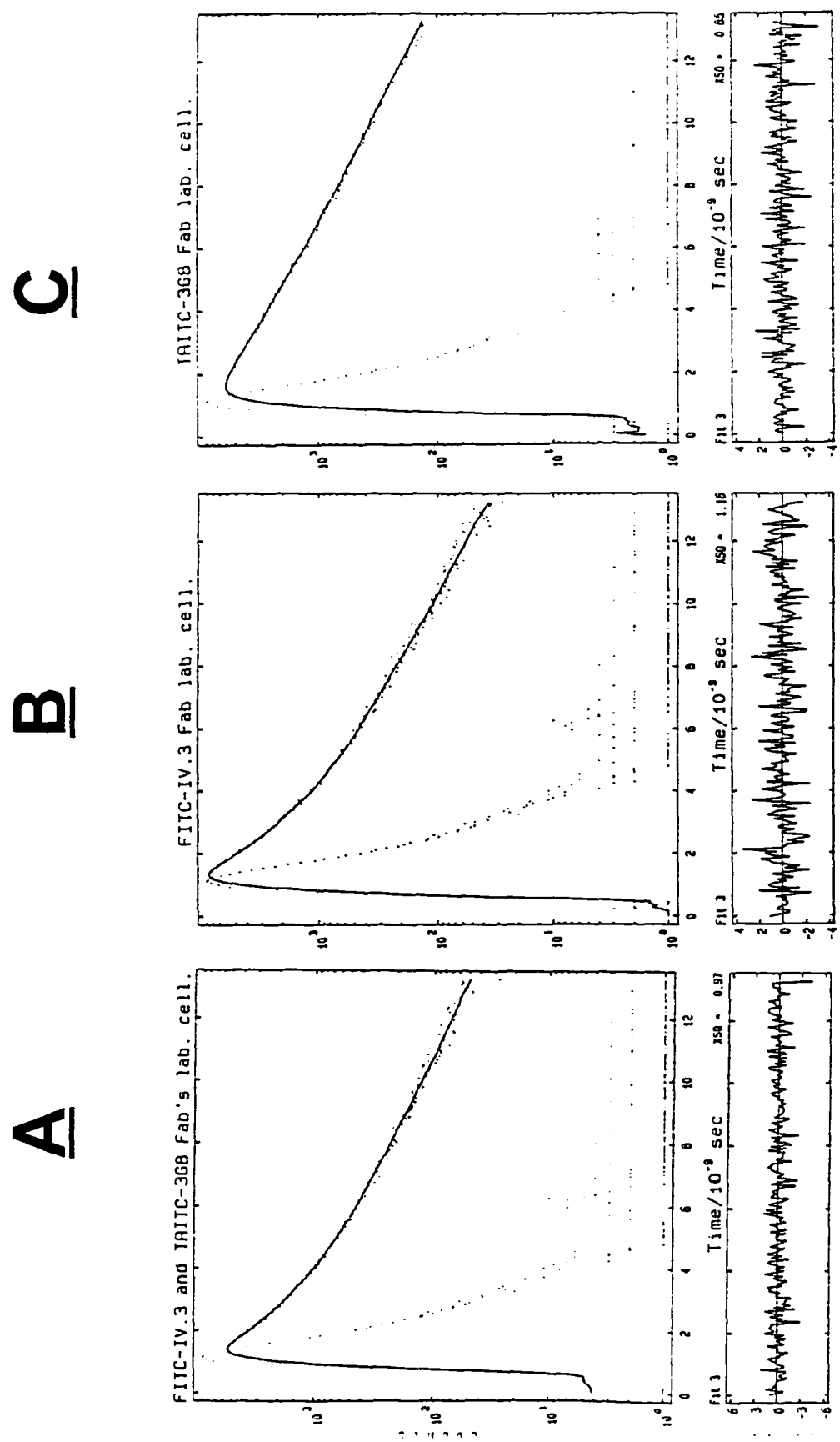


Figure 20. **Functional model for signaling by FcγRIIIB.** Clustering of GPI-anchored FcγR isoform leads to co-aggregation of membrane-spanning FcγR species. Juxtaposition of ITAM effectors in turn leads to initiation of the intracellular signal.

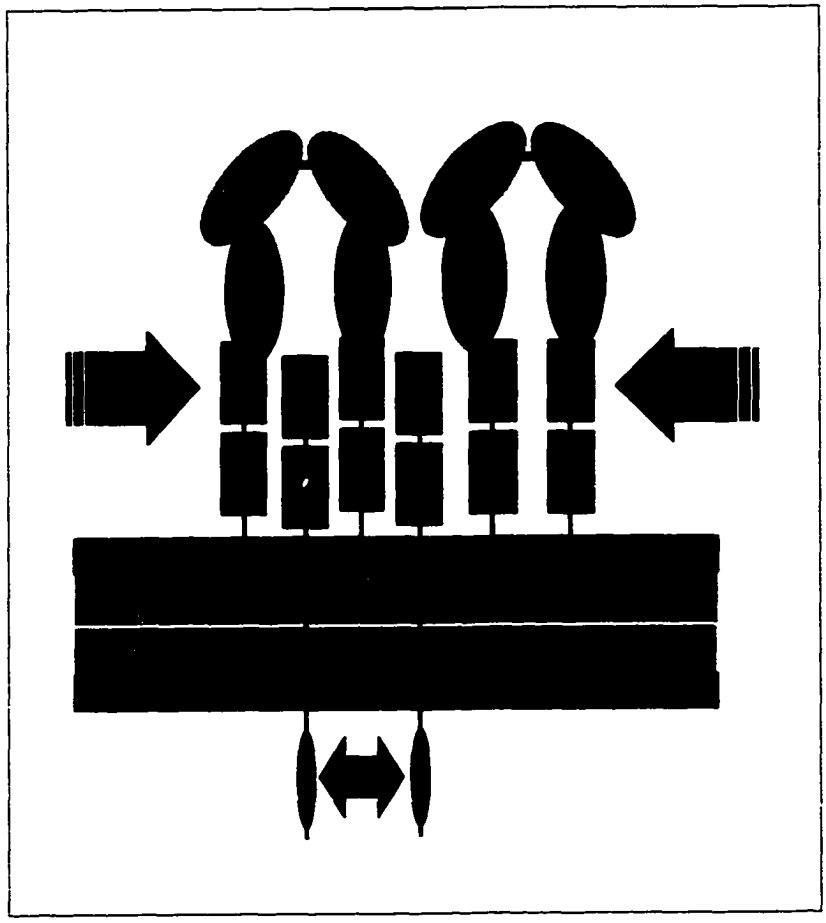
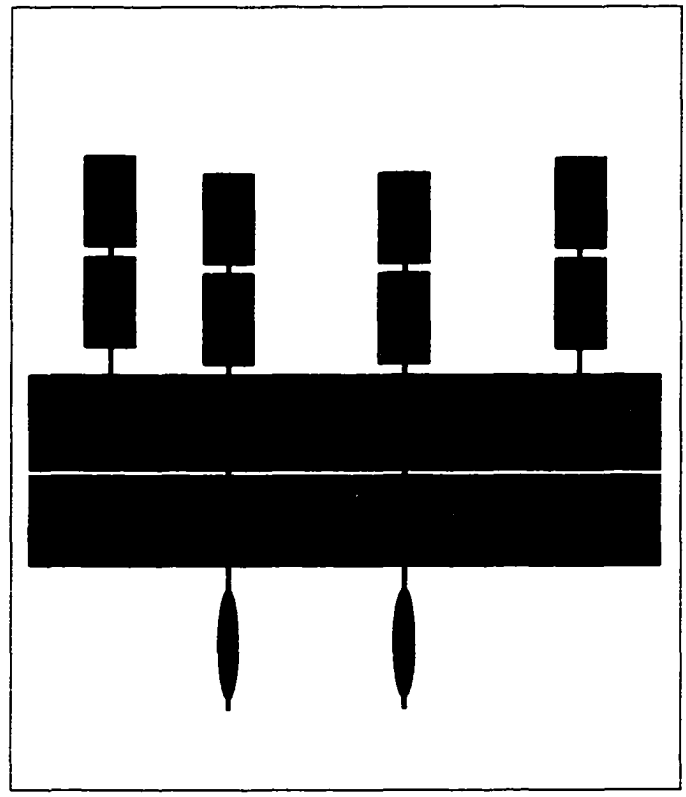


Figure 21. **Lateral organization of lipid bilayer, based on hydrophobic chain length matching.** Given the heterogeneity of constituent phospholipids in biological membrane, the lipid bilayer may be better represented by leaflets which vary in their partial thickness. GPI-anchored proteins containing long lipid chains might then be expected to co-localize with phospholipids of similar chain length.



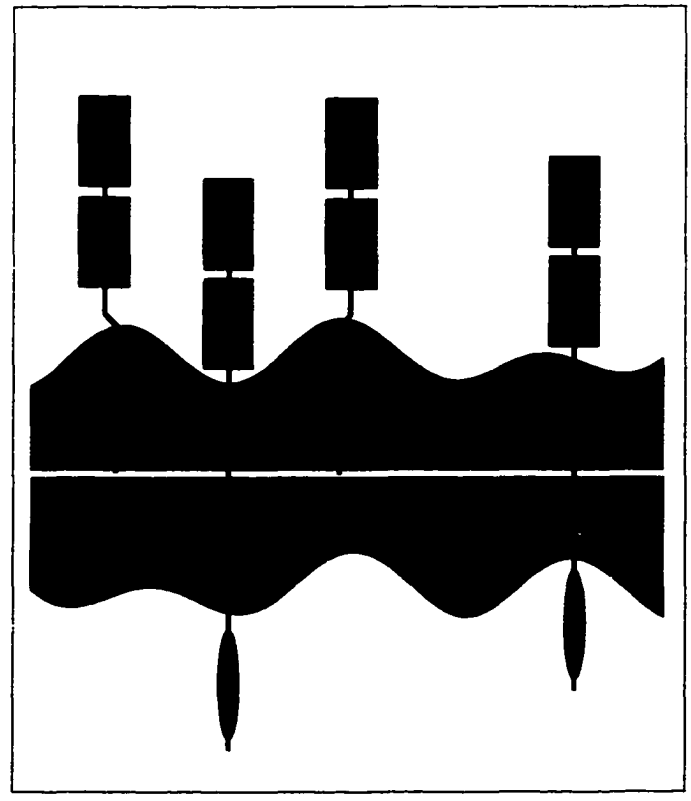


Figure 22. **DiI co-localization with FcγRIIIB.** Sets of paired fluorescence images showing the effect of FcγRIIIB crosslinking on the re-distribution of various chain-length derivatives of the DiI lipophilic probe. (see Methods) Although all three cells selected for illustration show evidence of DiI migration with crosslinked FcγRIIIB, the effect was more pronounced in PMNs labeled with DiI-C_{16}. (middle set)

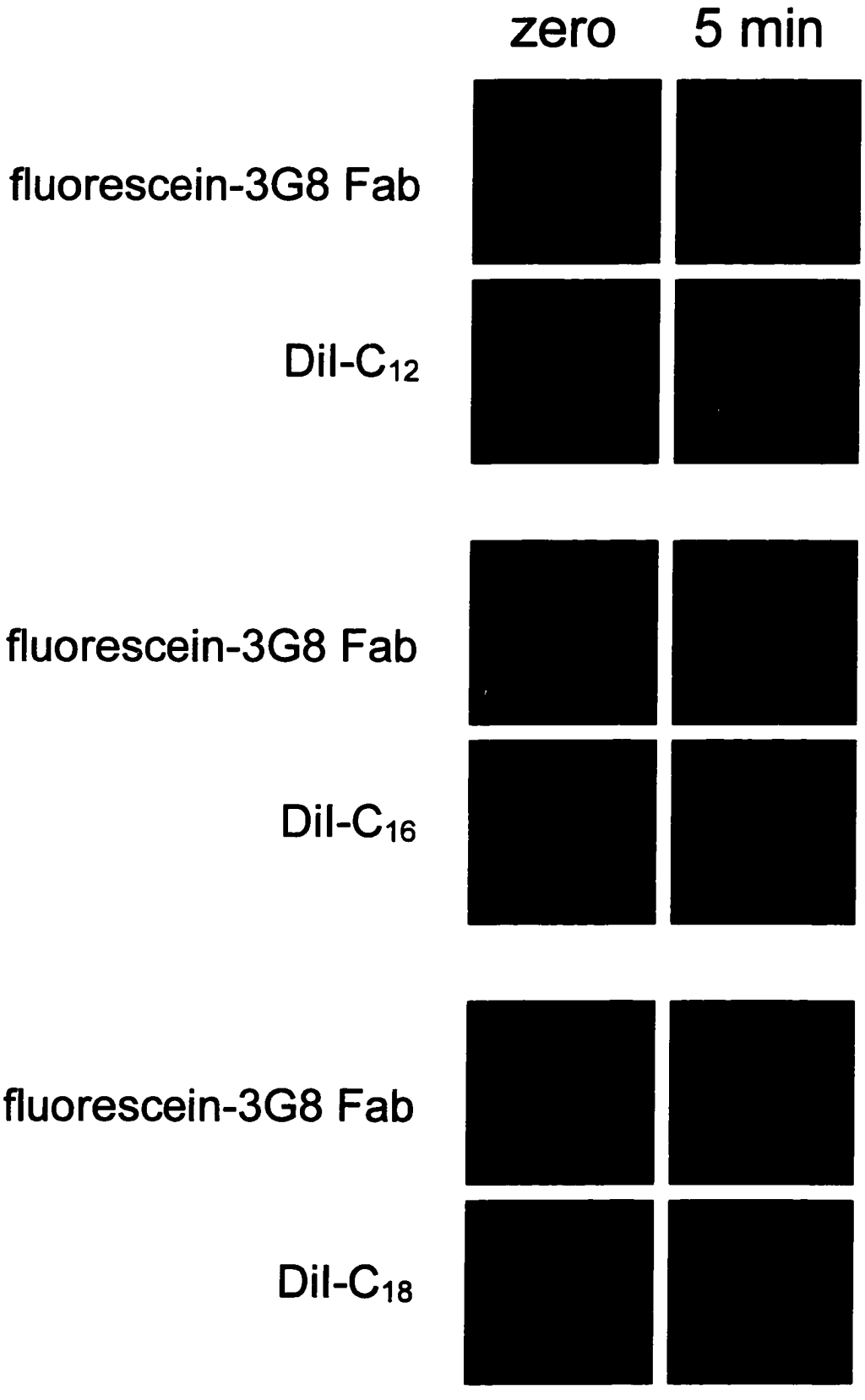


Figure 23. **"Signaling raft" model for transmembrane signaling by GPI-anchored proteins.** (extension of conceptual model proposed by Simons and Ikonen, [103]) Direct antibody crosslinking leads to formation of a membrane domain enriched in sphingosine-based lipids and cholesterol, drawing other signaling molecules (including transmembrane proteins and doubly-acylated *src* family kinases) to transduce signals across the lipid bilayer. The physical parameter which defines the lipid composition around the cluster of GPI-anchored proteins is uncertain, but is depicted here as the hydrophobic chain length.

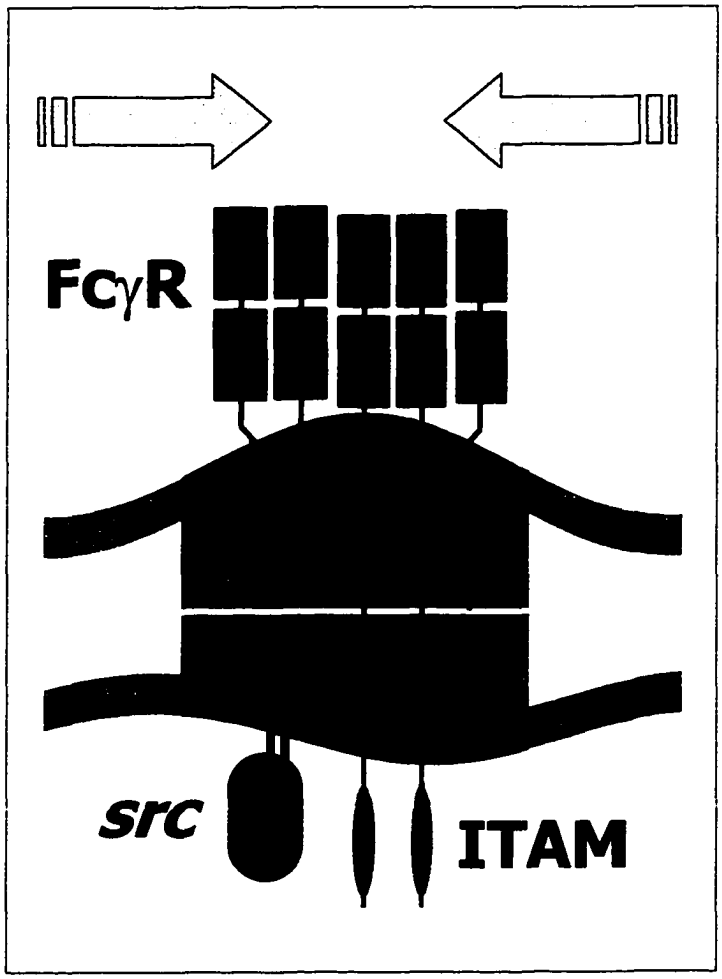


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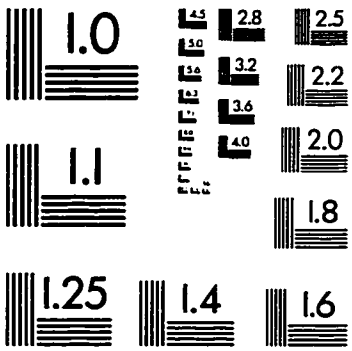
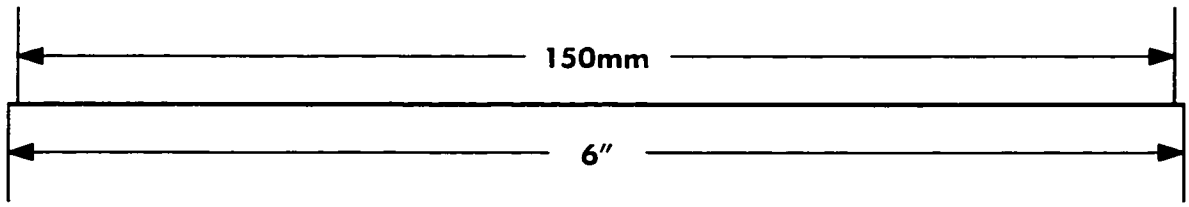
IMAGE EVALUATION TEST TARGET (QA-3)





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