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FcγRECEPTOR III INDUCES ACTIN POLYMERIZATION IN HUMAN NEUTROPHILS AND PRIMES PHAGOCYTOSIS MEDIATED BY FcγRECEPTOR II[1]

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Human polymorphonuclear leukocytes (PMN) express two classes of FcγR: FcγRII the 42-kDa receptor with a traditional membrane spanning domain and cytoplasmic tail and $Fc\gamma RIII_{PMN}$ the 50- to 80-kDa receptor with a glycosyl-phatidylinositol membrane anchor expressed on PMN. To explore the capacity of $Fc\gamma RIII_{PMN}$ to generate intracellular signals, we have analyzed the ability of Fab and $F(ab')_2$ anti-FcγR mAb to induce actin filament assembly, a prerequisite for motile behaviors. Multivalent ligation of $Fc\gamma RIII_{PMN}$, independent of FcγRII, results in an increase in F-actin content that is $[Ca^{2+}]_i$ dependent. Multivalent ligation of FcγRII also initiates actin polymerization but uses a $[Ca^{2+}]_i$-independent initial pathway. In addition to providing a mechanism for $Fc\gamma RIII_{PMN}$ triggered effector functions, the increase in F-actin and $[Ca^{2+}]_i$ generated by $Fc\gamma RIII_{PMN}$ ligation also serves as a "priming" signal to modify PMN responses to other stimuli. Expermients using erythrocytes specifically coated with anti-FcγRII Fab demonstrate that cross-linking of $Fc\gamma RIII_{PMN}$ with anti-FcγRIII $F(ab')_2$ enhances phagocytosis mediated by FcγRII. Thus, $Fc\gamma RIII_{PMN}$, a glycosyl-phosphatidylinositol anchored protein, may contribute directly to an intracellular program of actin assembly that may trigger and prime neutrophil effector functions.

Human neutrophils (PMN)[3] participate in a range of cellular functions, including phagocytosis of immune complexes and opsonized particles, antibody-dependent cell-mediated cytotoxicity, and release of both reactive oxygen intermediates and specific granule proteins. Each of these functions can be triggered by FcγR. PMN possess two classes of FcγR: FcγRII that has a traditional membrane spanning domain and cytoplasmic tail (1–3) and $Fc\gamma RIII_{PMN}$ that has a GPI anchor (4–8). Because each receptor expresses a different but functionally significant structural polymorphism (9–11), recent studies have attempted to examine the relative roles of FcγRII and $Fc\gamma RIII_{PMN}$ in these functions (9, 12–23).

$Fc\gamma RIII_{PMN}$ has been considered primarily as a highly mobile binding molecule serving to present ligand to FcγRII for subsequent transmembrane signaling, because of its GPI linkage with the lack of an obvious mechanism to couple with guanine nucleotide-binding regulatory proteins or cytoskeletal structures. Although some evidence suggests an apparent inability to trigger certain effector functions (14–19), other evidence indicates that $Fc\gamma RIII_{PMN}$ may serve some functions independently of FcγRII. For example, Con A-coated rabbit E (E-Con A) are internalized by PMN through the obligatory participation of $Fc\gamma RIII_{PMN}$ (20). The observation that blockade of $Fc\gamma RIII_{PMN}$ by Fab fragments of the anti-FcγRIII mAb 3G8 blocks phagocytosis of E-Con A without decreasing E-Con A binding to PMN suggests that engagement of $Fc\gamma RIII_{PMN}$ may deliver a critical intracellular signal for phagocytosis. Indeed, multivalent ligation of $Fc\gamma RIII_{PMN}$ initiates both membrane depolarization and an increase in $[Ca^{2+}]_i$ (24). From among many possibilities, such a signal could indicate $[Ca^{2+}]_i$-dependent activation of kinases and other intracellular enzymes.

To explore the capacity of $Fc\gamma RIII_{PMN}$ to generate intracellular signals, we have analyzed the ability of anti-FcγR mAb to induce actin filament assembly in PMN. Shifts in actin from its globular monomeric form to its filamentous form, F-actin are a prerequisite for motile behaviors such as phagocytosis, secretion, and locomotion and often accompany "priming" of the PMN for enhanced responses to other stimuli (25–27). Our data indicate that multivalent ligation of $Fc\gamma RIII_{PMN}$, independent of FcγRII, results in a significant, $[Ca^{2+}]_i$-dependent increase in PMN F-actin content. Furthermore, using E specifically coated with anti-FcγRII Fab, our data demonstrate that cross-linking of $Fc\gamma RIII_{PMN}$ with anti-FcγRIII $F(ab')_2$ enhances phagocytosis mediated by FcγRII. Thus, $Fc\gamma RIII_{PMN}$, a GPI-anchored protein, contributes directly to actin filament assembly that may trigger and prime for neutrophil effector functions.

MATERIALS AND METHODS

Subjects. Peripheral blood was collected from disease-free volunteers by standard sterile venipuncture techniques. Protocols for these studies were approved by the Institutional Committee on Human Rights in Research.

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[2] Address correspondence and reprint requests to Dr. Jane E. Salmon, The Hospital for Special Surgery, 535 East 70th Street, New York, NY 10021.
[3] Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; Agg-IgG, aggregated human IgG; EA, bovine E opsonized with rabbit IgG; F-actin, filamentous actin; $Fc\gamma RIII_{PMN}$, the 50- to 80-kDa receptor for the Fc portion of IgG encoded by FcγRIII-1 gene and expressed on PMN; GAM, goat anti-mouse $F(ab')_2$ fragments; GPI, glycosyl-phosphatidylinositol; NBD, nitrobenzoxadiazole; E-GnA, Con A-coated bovine E.

Reagents. The chemotactic peptide, FMLP (Sigma Chemical Co., St. Louis, MO), was dissolved in ACS grade DMSO (Fisher Scientific, Pittsburgh, PA) for a stock concentration of 10^{-3} M and stored in sterile, pyrogen-free containers at −20°C. Aggregated IgG was prepared by heat aggregation of chromatographically purified 7S human IgG from Cohn fraction II (Miles Biochemicals, Elkhart, IL) (28). Soluble aggregates were sized by column chromatography with AcA22 (LKB, Rockville, MD) and stored in aliquots at −70°C. Sucrose-density gradient ultracentrifugation demonstrated predominantly complexes more than 19S. GAM IgG $F(ab')_2$, free of intact IgG by silver stain analysis of SDS-PAGE analytical gels, was obtained from Tago Immunochemicals (Burlingame, CA). GAM $F(ab')_2$ directly conjugated to FITC (Cappel Laboratories, Cochranville, PA) was used to confirm specific binding of this reagent to mouse mAb on the cell surface (24).

mAb. mAb 3G8 (mIgG1) recognizing FcγRIII (CD16) was a gift from Dr. Jay Unkeless (Mount Sinai Medical School, New York, NY) (29). The 3G8 Fab and 3G8 $F(ab')_2$ fragments and the mAb IV.3 (mIgG2b) and IV.3 Fab fragments recognizing FcγRII (CD32) (12) were obtained from Medarex, Inc. (West Lebanon, NH). Fab fragment preparations contained no detectable intact IgG or H chains, as judged by silver stain of SDS-PAGE analytical gels. In selected experiments mAb CIKM5 (mIgG1 recognizing CD32), a gift of Dr. G. R. Pilkington (University of Melbourne, Melbourne, Australia) was used as an intact IgG (30). mAb preparations were sterile and contained no detectable endotoxin as determined by the limulus amoebocyte assay (Associates of Cape Cod, Woods Hole, MA). Murine IgG1 myeloma protein (MOPC-21), an isotype control, was obtained from Sigma. Saturating concentrations of anti-receptor mAb and mAb fragments was determined by standard immunofluorescence techniques as previously described (8, 31).

Preparation of neutrophils. Peripheral blood was drawn into heparinized syringes. Neutrophils (PMN) were separated by discontinuous two-step centrifugation on Ficoll-Hypaque density gradients (d = 1.077 and 1.119) followed by hypotonic lysis of contaminating E (9). Microscopic examination of the cells demonstrated >98% PMN. Separations were completed within 90 min and all experimental procedures were done within 4 h after phlebotomy.

In some experiments intracellular calcium was chelated by incubating cells in PBS (125 mM NaCl, 10 mM PO_4 with 5 mM KCl and 5 mM glucose) with 50 μM BAPTA-acetoxymethyl ester (Molecular Probes, Junction City, OR), a non-fluorogenic calcium chelator, at 37°C for 30 min. PMN were incubated with modified Hanks'/HEPES (2.65 mM $MgCl_2$ and 2.0 mM $CaCl_2$) for 10 min at 37°C and then exposed to triggering stimuli. We have previously shown that preloading cells with BAPTA abolishes the change in $[Ca^{2+}]_i$ induced by FMLP or mAb 3G8 (24).

Stimulation of neutrophils. Freshly isolated PMN were resuspended (2.2×10^6/ml) in Hanks' balanced salt solution/HEPES buffer (138 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 1 mM $CaCl_2$, 4 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 25 mM HEPES). Aliquots of PMN were incubated with stimuli or control buffer for various times at 37°C. The control for FMLP included the appropriate concentration of DMSO. All mAb and Fab fragments were used at saturating concentrations as determined by indirect immunofluorescence and flow cytometry (31). In some experiments, cells were preincubated with anti-FcγR mAb or their fragments for 5 min at 37°C, washed, and subsequently incubated with GAM $F(ab')_2$ for cross-linking.

Staining of F-actin with NBD-Phallicidin. After stimulation, the PMN were stained with NBD-phallacidin (Molecular Probes) according to the method of Howard and Meyer (32) and analyzed by flow cytometry. Briefly, PMN (0.9 ml at 2.2×10^6 cells/ml in Hanks'/HEPES) were fixed, permeabilized and stained by the addition of 0.1 ml of 37% phosphate-buffered formalin containing 100 μg lysophosphatidylcholine (Avanti Polar Lipids, Pelham, AL) and 1.65×10^{-6} M NBD-phallacidin. After incubation for 10 min at 37°C, the cells were centrifuged at 400 × *g* for 5 min at room temperature and resuspended in Hanks'/HEPES. Microscopic examination of representative samples of stimulated stained PMN revealed no aggregates.

Quantitation of F-actin content by flow cytometry. Flow cytometric analysis of NBD fluorescence in PMN was performed within 1 h of staining. Cell-associated fluorescence was quantitated on a Cytofluorograf IIs equipped with a 3.5 decade logarithmic amplifier and a 2151 computer (Becton Dickinson Immunocytometry Systems, Westwood, MA). Exciting light at 488 nm was provided by an argon-ion laser. NBD emission fluorescence was collected through a 510-nm long pass filter. Before each experiment, the instrument was calibrated with FITC-conjugated calf thymus nuclei (Fluorotrol-GF, Becton Dickinson). PMN were identified by characteristic forward and right angle scattering. Histograms plotting cell number vs fluorescence intensity (in arbitrary units) were recorded for each sample and yielded normal distributions with one population of responding cells. Linear fluorescent data were used to compare F-actin content in stimulated and unstimulated cells. Relative F-actin content was expressed as the ratio of the mean linear fluorescence intensity of stimulated PMN to the mean linear fluorescence intensity of control cells. The relative F-actin content as determined by flow microfluorimetry analysis of NBD-phallicidin stained PMN has been shown to correlate with cellular content of F-actin (32, 33).

Antibody-sensitized E rosettes. EA, prepared by incubating bovine E with rabbit IgG anti-bovine E antibody (Cappel Laboratories, Cochranville, PA) for 1 h at 37°C, were resuspended at 10^8 cells/ml in RPMI and 20% FCS. The assessment of EA rosettes was performed as described previously (20).

Quantitation of FcγR expression by flow cytometry. FcγR expression on freshly isolated neutrophils was quantitated by indirect immunofluorescence. PMN (5×10^5 cells in PBS with 0.1% FCS) were incubated with saturating doses of mAb 3G8 or IV.3 at 4°C for 30 min and, after washing twice with iced PBS-FCS, were further incubated with saturating concentrations of FITC-conjugated rabbit anti-mouse IgG $F(ab')_2$ fragments (Cappel). Cell-associated immunofluorescence was quantitated in a Cytofluorograf IIs. Data were recorded and displayed in log fluorescence units.

Assay of phagocytosis. Quantitation of PMN FcγR-mediated phagocytosis was performed as previously described (9, 20). E coated with anti-FcγRII mAb (E-IV.3) were prepared by incubating bovine E with an equal volume of heteroantibodies of Fab IV.3 covalently linked to $F(ab')_2$ anti-ox E antibodies (generously provided by Dr. Li Shen of Dartmouth Medical School, Hanover, NH) for 18 h at 4°C with gentle mixing (19, 34). After washing, E-IV.3 were resuspended as a 2% suspension in RPMI and 20% FCS. In preliminary studies using E-IV.3 in our assay of phagocytosis, monovalent IV.3 Fab (40 μg/ml) blocked the internalization of the multivalent E-IV.3 (96% inhibition). These data, coupled with previously published work (14, 19, 21, 34) and the observation that 3G8 $F(ab')_2$ did not inhibit E-IV.3 internalization, indicate that E-IV.3 is an FcγRII-specific probe.

To assess internalization of E target particles, PMN in RPMI with 20% heat-inactivated IgG-free FCS (GIBCO, Grand Island, NY) at 5×10^6 cells/ml were preincubated with either 3G8 $F(ab')_2$ (5 μg/ml) or control medium for 2 min at 37°C. The mAb were present throughout the assay of phagocytosis. PMN (0.1 ml) were combined with EA (0.1 ml) or E-IV.3 (0.1 ml). The PMN-E mixtures were centrifuged at 44 × *g* for 3 min and then incubated at 37°C for 30 min. After lysis of non-internalized E with ammonium chloride, phagocytosis was quantitated by light microscopy. At least 400 cells per slide were counted in duplicate without knowledge of the preincubation condition. The data are expressed as percent phagocytosis (%P: % of PMN with one or more internalized erythrocyte).

Data analysis. The values for relative F-actin content represent the mean ± SD. The percent of control phagocytosis in experiments using 3G8 $F(ab')_2$ as a "priming" stimulus was calculated as: $(\text{phagocytosis}_{3G8\ F(ab')2} - \text{phagocytosis}_{control})$ divided by $\text{phagocytosis}_{control}$. Phagocytic capacity of PMN pre-treated with 3G8 $F(ab')_2$ was compared to that of control PMN using a Wilcoxon matched-pairs signed-ranks test. For all statistics a probability of 0.05 was used to reject the null hypothesis that there is no difference between the groups.

RESULTS

Neutrophil F-actin content is increased by aggregated IgG. Multivalent IgG, presented as a soluble aggregate or as an opsonized particle, is an effective trigger of phagocytosis and other effector functions in PMN. Accordingly, we examined the capacity of heat Agg-IgG, the native ligand for both FcγRII and $FcγRIII_{PMN}$, to trigger actin filament assembly. PMN were incubated with Agg-IgG (100 μg/ml) at 37°C for various periods of time. As shown in Figure 1, the relative F-actin content showed a biphasic increase with Agg-IgG. Within 15s F-actin content was 1.77 ± 0.54 (mean ± SD) fold higher in Agg-IgG-stimulated PMN. After a slight decrease evident at 1 min (1.52 ± 0.20), F-actin progressively increased reaching a maximum relative F-actin content of 2.13 ± 0.30 at 5 min. The increase persisted beyond 10 min. Concentrations of Agg-IgG as low as 12.5 μg/ml resulted in similar levels and kinetics of response (1.63 at 2 min). The increase in F-actin induced by Agg-IgG was of similar magnitude and was as rapid as that seen for a maximum

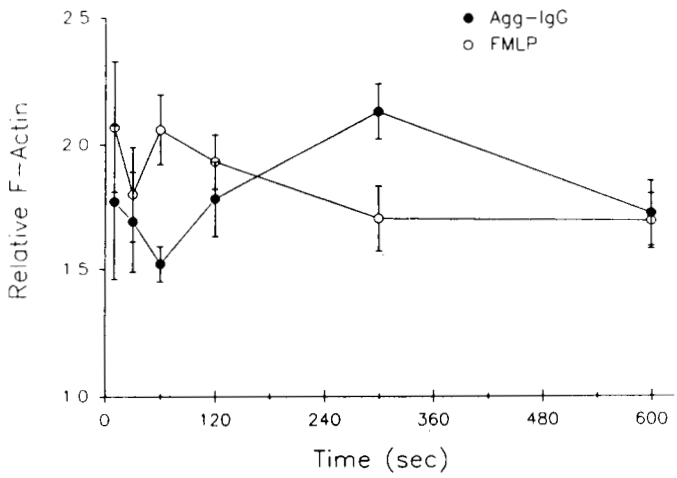

Figure 1. Agg-IgG induces actin polymerization. PMN (2.2×10^6/ml) were incubated with Agg-IgG (100 μg/ml) or control buffer or FMLP (5×10^{-9} M) or the appropriate concentration of DMSO at 37°C. At the indicated times after stimulation cells were stained with NBD-phallicidin and analyzed by flow microfluorimetry for quantitation of F-actin content. Relative F-actin content was expressed as the ratio of the mean linear fluorescence intensity of stimulated PMN to the mean linear fluorescence intensity of control cells. The values represent the mean ± SD for three to nine different experiments. Concentrations of Agg-IgG as low as 12.5 μg/ml gave similar values for F-actin and kinetics of response. Stimulation with FMLP (10^{-6} to 10^{-9} M) resulted in similar approximately twofold increases in F-actin content, as demonstrated by Howard and Oresajo (33).

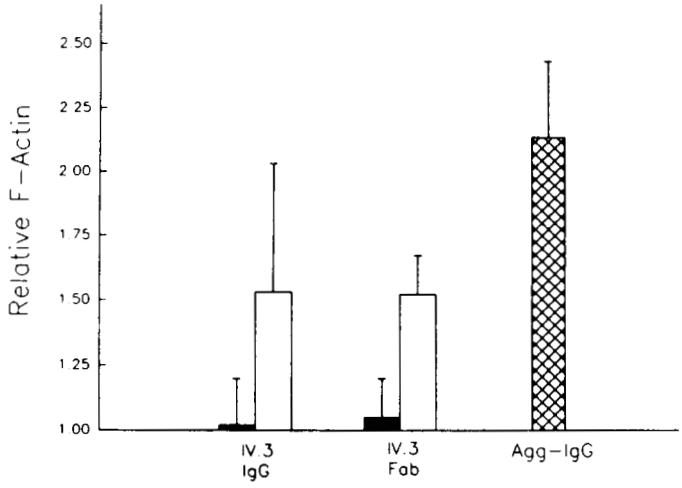

Figure 2. Cross-linking of FcγRII initiates actin polymerization. PMN were incubated with: ■, mAb IV.3 IgG or IV.3 Fab (5 μg/ml) for 5 min at 37°C; ▩, Agg-IgG for 5 min; and □, mAb IV.3 IgG or IV.3 Fab for 2 min, followed by GAM F(ab')$_2$ for 5 min. Thereafter, cells were stained and F-actin was quantitated described in Figure 1. Values represent the means ± SD for four to seven different experiments.

response to FMLP (Fig. 1).

Cross-linking of FcγRII can induce rise in F-actin content. Inasmuch as FcγRII in PMN can mediate phagocytosis without simultaneous ligation of FcγRIII$_{PMN}$ (19), we hypothesized that ligation of FcγRII should initiate actin filament assembly. Using the mAb anti-FcγRII mAb IV.3 IgG at saturating concentrations (5 μg/ml), we were unable to trigger an increase in F-actin (Figs. 2 and 3*A*). Experiments with mAb CIKM5 IgG, another anti-FcγRII mAb, however, did result in actin polymerization (relative F-actin content = 1.43 ± 0.41, n = 3) and cross-linking IV.3 IgG with GAM F(ab')$_2$ (50 μ/ml) did elicit a rise in F-actin content (Fig. 2).

To establish whether the increase in F-actin content initiated by anti-FcγRII mAb required formation of heterotypic clusters of FcγRII and FcγRIII$_{PMN}$ via the Fc region of the mAb, we opsonized PMN with IV.3 Fab fragments followed by cross-linking with GAM F(ab')$_2$. Figure 2 shows univalent ligation of FcγRII was ineffective but that clusters of FcγRII can initiate actin assembly (relative F-actin content = 1.52 ± 0.15), a response that persisted beyond 5 min. Thus, the lack of response with monomeric mAb IV.3 IgG appears to reflect unique binding properties of either the mAb or its Fc piece, rather than an inability of FcγRII per se to trigger the actin polymerization.

FcγRIII$_{PMN}$ can trigger actin filament assembly. The ability of FcγRII to mediate actin polymerization raised the possibility that this receptor was responsible for the Agg-IgG-induced response. To explore the possibility that the GPI-anchored FcγRIII$_{PMN}$ might elicit a similar response, we used the anti-FcγRIII mAb 3G8 to ligate FcγRIII$_{PMN}$. PMN incubated with mAb 3G8 IgG (2 μg/ml) had an increase in F-actin that reached a peak by 5 min (1.55 ± 0.17; n = 11; Fig. 3*A*). The rise in F-actin persisted beyond 10 min. Concentrations of 3G8 IgG ranging from

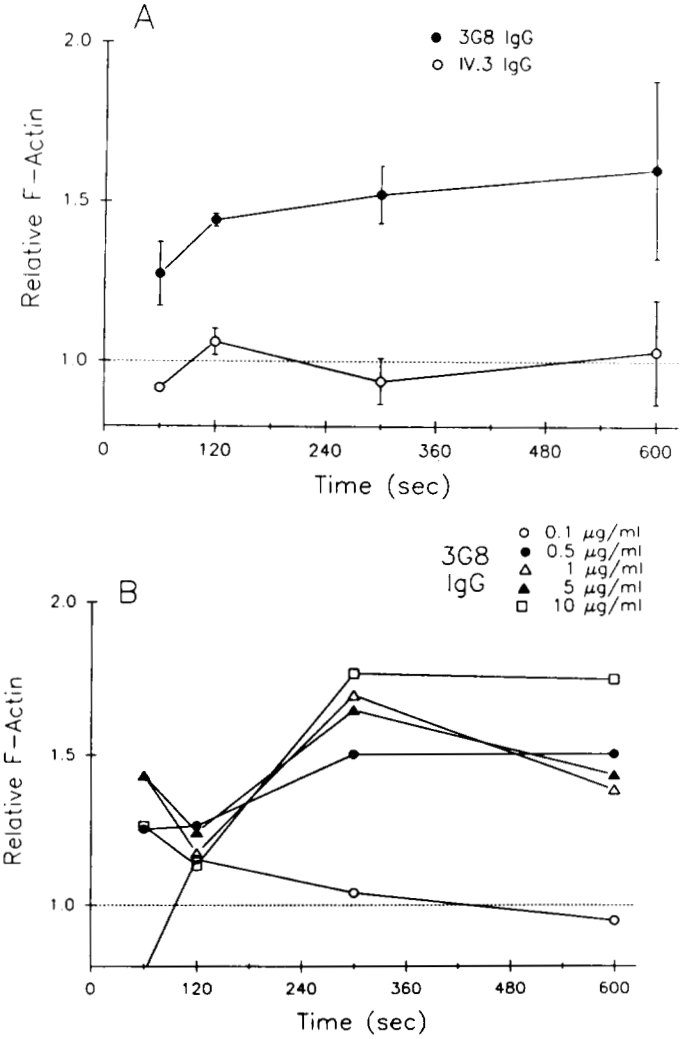

Figure 3. FcγRIII$_{PMN}$ ligation triggers actin polymerization. *A*, PMN were incubated with mAb IV.3 IgG or 3G8 IgG for the indicated times. Relative F-actin content was determined as described in Figure 1. Values represent mean ± SD of three to six experiments. *B*, Dose response comparing the increase in F-actin content to the concentration of mAb 3G8 IgG used to stimulate PMN.

0.5–10 μg/ml gave similar responses (Fig. 3*B*).

Univalent ligation of $Fc\gamma RIII_{PMN}$ with saturating concentrations of 3G8 Fab (5 to 10 μg/ml) did not increase F-actin (relative F-actin = 0.97 ± 0.8, n = 3; Figs. 4 and 5). In contrast, in each of four experiments with 3G8 $F(ab')_2$, there was an increase in F-actin content suggesting that bivalent crosslinking of $Fc\gamma RIII_{PMN}$ is a sufficient stimulus to trigger assembly of filamentous actin (F-actin = 1.18 ± 0.12; Fig. 5). The addition of GAM $F(ab')_2$ (50 μg/ml) to further cross-link $Fc\gamma RIII_{PMN}$ previously ligated with 3G8 Fab or $F(ab')_2$ resulted in a dramatic rise in F-actin, larger in magnitude than that for 3G8 alone (after 5 min at 37°C relative F-actin content: 3G8 Fab + GAM, 2.05 ± 0.39, n = 3; 3G8 $F(ab')_2$ + GAM, 1.87 ± 0.28, n = 4; and 3G8 IgG, 1.50 ± 0.29, n = 4; Fig. 5). The actin polymerization induced by this selective ligation and clustering of only $Fc\gamma RIII_{PMN}$ with 3G8 Fab and GAM $F(ab')_2$ demonstrates the capacity of $Fc\gamma RIII_{PMN}$ to initiate cytoskeletal rearrangement without the obligatory participation of FcγRII.

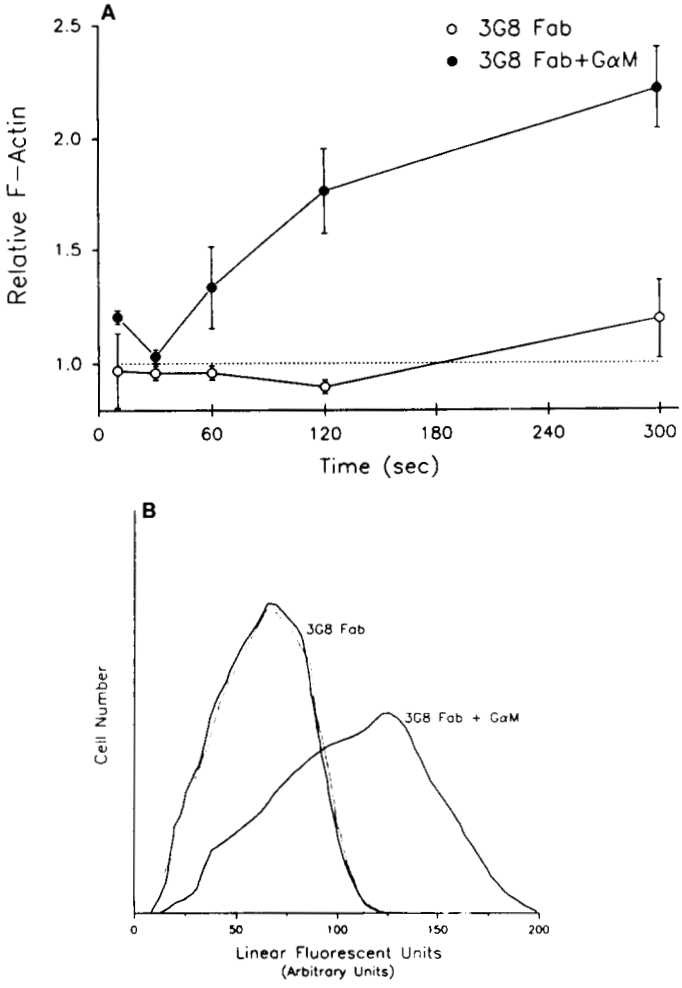

Figure 4. *A*, Kinetics of F-actin assembly induced by 3G8 Fab crosslinked with GAM $F(ab')_2$. PMN were opsonized with 3G8 Fab for 2 min and then incubated with GAM $F(ab')_2$ or control medium for the indicated times. Values represent means ± SD of three experiments. *B*, Histogram of a representative experiment measuring relative F-actin content in PMN stimulated with 3G8 Fab cross-linked with GAM $F(ab')_2$. PMN were incubated with 3G8 Fab followed by GAM $F(ab')_2$ or control buffer (——) for 5 min. Control cells were incubated with mIgG1 followed by control buffer (– – –). There was a 1.57-fold increase in F-actin content after crosslinking 3G8 Fab ($MFI_{3G8 + G\alpha M}/MFI_{3G8}$ = 105.7/60.9). The 3G8 Fab alone was not different from the mIgG1 control.

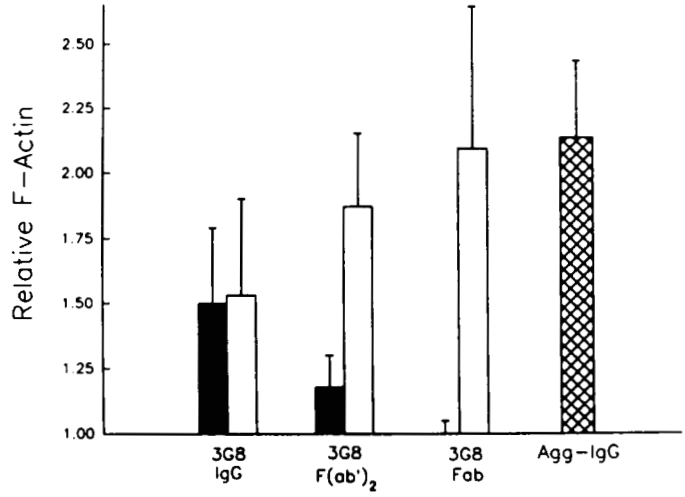

Figure 5. $Fc\gamma RIII_{PMN}$ can independently initiate F-actin assembly: the relationship of valency of ligation to initiation actin polymerization. PMN were incubated with: ■, mAb 3G8 IgG, 3G8 $F(ab')_2$, or 3G8 Fab (5 μg/ml) for 5 min at 37°C; ▨, Agg-IgG for 5 min; □, mAb 3G8 IgG, 3G8 $F(ab')_2$ or 3G8 Fab for 2 min, followed by GAM $F(ab')_2$ for 5 min. Thereafter, cells were stained and F-actin was quantitated described in Figure 1. Values represent the means ± SD for four to seven different experiments.

The kinetics of the filamentous actin assembly induced by 3G8 Fab crosslinked with GAM $F(ab')_2$ (Fig. 4*A*) are similar to the response initiated by both 3G8 IgG (Fig. 3*A*) and Agg-IgG (Fig. 1). Interestingly, additional cross-linking of mAb on cells previously treated with 3G8 IgG (2 μg/ml for 5 min at 37°C) with saturating doses of GAM $F(ab')_2$ (50 μg/ml for 5 min at 37°C) did not lead to a further increase in relative F-actin content (3G8 IgG vs 3G8 IgG + GAM: 1.50 ± 0.29 vs 1.53 ± 0.37; Fig. 5). Similar secondary cross-linking of 3G8 $F(ab')_2$ led to an increased level of F-actin but one that did not reach the level achieved with 3G8 Fab as the primary anti-$Fc\gamma RIII_{PMN}$ reagent. These observations suggest that crosslinking of $Fc\gamma RIII_{PMN}$ with mAb 3G8 may make PMN relatively refractory to further cumulative actin polymerization by the same receptors. The magnitude of response appears to be dependent on the degree of cross-linking and the temporal sequence of cross-linking signals.

$Fc\gamma RIII_{PMN}$-induced rise in F-actin content is $[Ca^{2+}]_i$ dependent. Although cross-linking of FcγRII by mAb IV.3 and of $Fc\gamma RIII_{PMN}$ by mAb 3G8 each leads to F-actin formation, the same cross-linking leads to divergent responses in $[Ca^{2+}]_i$ fluxes (24). In PMN such divergent relationships between changes in $[Ca^{2+}]_i$ and induction of actin polymerization have been observed with various triggering stimuli. For example, the increase in F-actin induced by the chemotactic peptide FMLP is not dependent on simultaneous changes in $[Ca^{2+}]_i$ (35–39), whereas that induced by adherence involves a calcium-dependent pathway (26). Therefore, we examined the possibility that FcγRII and $Fc\gamma RIII_{PMN}$ might use two different, and perhaps complementary, transmembrane signaling pathways to induce actin polymerization. Based on the previously defined properties of the individual mAb (24), we postulated that $Fc\gamma RIII_{PMN}$, ligated by 3G8, might use a $[Ca^{2+}]_i$-dependent pathway and that FcγRII, ligated by IV.3, might use a $[Ca^{2+}]_i$-independent pathway. Accordingly, PMN were loaded with BAPTA acetoxymethylester (50 μM/ml), a nonfluorgenic Ca^{2+} chelator, for 30 min at

37°C. BAPTA pretreatment blocks >95% of the $[Ca^{2+}]_i$ response to receptor ligation (24) but does not change FcγR expression as assessed both by flow cytometric analysis with mAb 3G8 and IV.3 and by EA binding. In PMN pretreated with BAPTA, 3G8 IgG-induced actin assembly was markedly reduced. The later phase of actin polymerization was completely blocked (Fig. 6*A*). As expected, the early increase in F-actin content in response to FMLP (which is not dependent upon a simultaneous increase in $[Ca^{2+}]_i$ (35–39)) was preserved in BAPTA-treated cells (Fig. 6*B*). Interestingly, F-actin content at 5 min after FMLP stimulation remained higher in BAPTA-treated cells supporting the observations of Downey et al. (39) that elevated $[Ca^{2+}]_i$ facilitates actin disassembly.

To pursue the suggestion that $Fc\gamma RIII_{PMN}$-induced actin polymerization is $[Ca^{2+}]_i$-dependent, selective cross-linking of $Fc\gamma RIII_{PMN}$ by Fab fragments was analyzed. BAPTA markedly reduced the increase in F-actin content initiated by 3G8 Fab and GAM $F(ab')_2$ by 41 ± 17% and 68 ± 9%, at 2 and 5 min, respectively (n = 5). In contrast, the response initiated by IV.3 Fab followed by GAM $F(ab')_2$ was less sensitive to chelation of intracellular $[Ca^{2+}]_i$. The increase in F-actin content triggered by FcγRII clustering in BAPTA-treated cells was not blocked (110 ± 23% control) 2 min after cross-linking with GAM $F(ab')_2$ and was only decreased 29 ± 29% at 5 min (n = 5). Although inhibition by BAPTA appeared to be less for crosslinked 3G8 Fab than for 3G8 IgG, perhaps because the former is a more potent stimulus, the data suggest two different signaling pathways, with the response induced by $Fc\gamma RIII_{PMN}$ more dependent upon an increase in $[Ca^{2+}]_i$ than that induced by FcγRII.

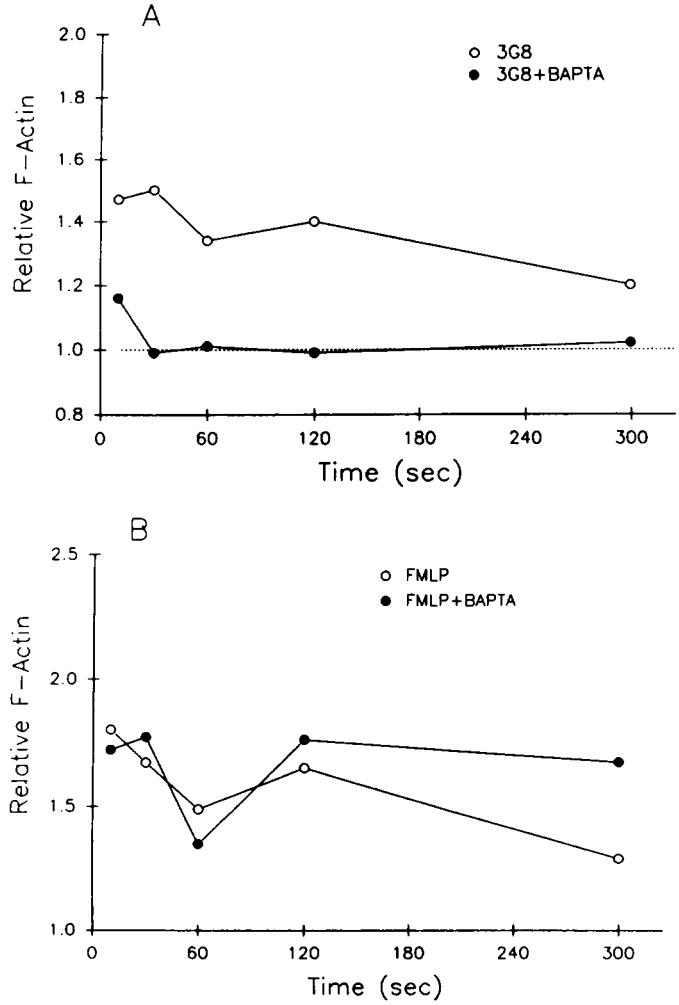

Figure 6. $Fc\gamma RIII_{PMN}$ induced actin polymerization is $[Ca^{2+}]_i$-dependent. Intracellular calcium was chelated with BAPTA (50 μg/ml) for 30 min. Thereafter, cells were stimulated with 3G8 IgG (*A*) or FMLP (5×10^{-9} M) (*B*) or the appropriate control medium.

Cross-linking of $Fc\gamma RIII_{PMN}$ as priming event. In PMN increases in $[Ca^{2+}]_i$ and actin filament assembly, both of which can be induced by cross-linking of $Fc\gamma RIII_{PMN}$, have been associated with enhancement or "priming" of effector functions (40–44). We considered the possibility, therefore, that cross-linking of $Fc\gamma RIII_{PMN}$ might "prime" the PMN response of other receptors. Inasmuch as ligation of $Fc\gamma RIII_{PMN}$ with 3G8 $F(ab')_2$ is sufficient to induce a rise in F-actin, we tested this hypothesis by examining the effect of pretreatment of PMN with 3G8 $F(ab')_2$ (37°C for 2 min) on the FcγRII-mediated phagocytosis of E opsonized with anti-FcγRII Fab (E-IV.3). Based on blocking studies (see *Materials and Methods*) and other data (14, 19, 21, 34), E-IV.3 is an FcγRII-specific probe of phagocytosis. In six of six experiments, each using PMN from a different donor, there was an increase in FcγRII-mediated phagocytosis by the PMN pretreated with 3G8 $F(ab')_2$ (Fig. 7). The average phagocytic capacity of the 3G8 $F(ab')_2$-treated PMN was 126 ± 27% of control ($p < 0.03$). Despite the fact that 3G8 $F(ab')_2$ delivers a submaximal stimulus, these data indicate that $Fc\gamma RIII_{PMN}$ can "prime" PMN to have an enhanced phagocytic response to engagement of FcγRII, independent of shared binding of ligand.

DISCUSSION

Human PMN express two classes of FcγR, FcγRII, and $Fc\gamma RIII_{PMN}$. Although FcγR can participate in a range of effector functions, the contributions of each receptor class remain incompletely defined. Inasmuch as $Fc\gamma RIII_{PMN}$ is a glycosyl-phosphatidylinositol-linked protein with the potential for rapid lateral mobility in the plasma membrane but with no obvious mechanism for signal transduction, this receptor has been considered primarily a binding molecule for multivalent ligand. Such bound ligand might be more effectively focused for interaction with FcγRII that can initiate antibody-dependent cell-mediated cytotoxicity, superoxide generation, release of granule proteins, and phagocytosis (13–16, 18, 19, 23). Several observations, however, suggest that $Fc\gamma RIII_{PMN}$ is more than a focusing molecule for FcγRII. Cross-linking of $Fc\gamma RIII_{PMN}$ initiates a membrane potential changes and a flux in $[Ca^{2+}]_i$ (24), and engagement of $Fc\gamma RIII_{PMN}$ appears to deliver a critical intracellular signal for phagocytosis of Con A-treated rabbit E (20). Despite its inability to lyse tumor targets (14, 17), $Fc\gamma RIII_{PMN}$ can lyse chicken E (13) and release granule proteins (23). The capacity for cross-linking of this receptor to initiate actin filament assembly (Figs. 4 and 5) by $[Ca^{2+}]_i$-dependent pathways apparently distinct from those activated by FcγRII (Fig. 6) may underlie some of these functions and contribute to the ability of $Fc\gamma RIII_{PMN}$ to facilitate FcγRII function (Fig. 7).

Aggregated IgG and immune complexes have previously been shown to induce actin assembly in PMN, but the capacity of each of the two classes of FcγR to initiate this response independently has not been explored (45, 46).

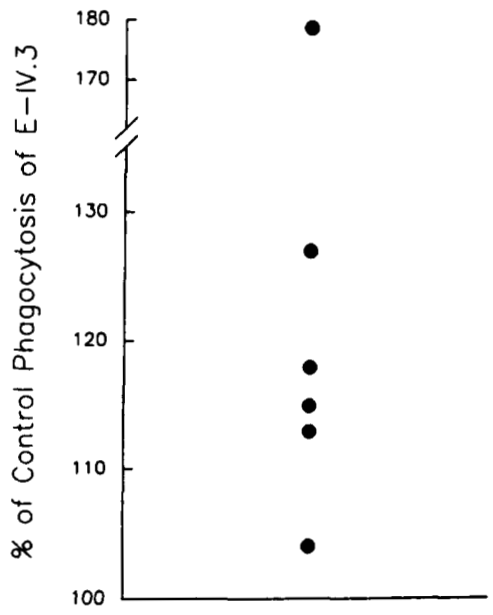

Figure 7. Bivalent ligation of $Fc\gamma RIII_{PMN}$ enhances FcγRII-mediated phagocytosis. PMN pre-treated with 3G8 $F(ab')_2$ (5 μg/ml) or control medium for 2 min were incubated with E-IV.3, an FcγRII-specific probe. After 30 min at 37°C, noninternalized E were lysed and phagocytosis was quantitated by light microscopy. The results, expressed as percent control phagocytosis, represent $(\%P_{3G8F(ab')2} - \%P_{control}) / \%P_{control}$ in each of six experiments from different donors. The average phagocytic capacity of 3G8 $F(ab')_2$-treated PMN was 126 ± 26% (mean ± SD) of control ($p < 0.03$).

Although the mechanism whereby $Fc\gamma RIII_{PMN}$ with its GPI anchor couples to guanine nucleotide-binding regulatory proteins or cytoskeletal structures is unknown, other GPI-anchored, such as TAP and Thy-1, mediate both transmembrane signaling and cytoskeletal rearrangement (47–51). These functions apparently depend on interactions with components of the TCR/CD3 complex (52–56). The observation that other integral membrane proteins may facilitate the linkage of Thy-1 with cytoskeletal elements (52), coupled with the coprecipitation of $Fc\gamma RIII_{PMN}$ and actin in PMN (57), suggests that $Fc\gamma RIII_{PMN}$ may also function as part of a multimolecular complex. Although a family of homologous proteins (e.g., ζ-chain of CD3 and the FcεRI γ-chain) has been shown to associate with FcγRIII in human NK cells (58–62), the identity of molecule(s) associated with $Fc\gamma RIII_{PMN}$ is not yet established. Indeed, an alternative mechanism for signal transduction might be receptor internalization and hydrolysis of the PI anchor with generation of either diacylglycerol or phosphatidic acid (63, 64). Whatever the mechanism, however, the capacity for $Fc\gamma RIII_{PMN}$ to initiate certain cell responses, including cytoskeletal assembly independently of FcγRII, is now established. A similar independent capacity for FcγRII is also clear.

Although the exact mechanism that triggers signal transduction and cytoskeletal rearrangement by $Fc\gamma RIII_{PMN}$ is unknown, it appears to differ from the pathways used by FcγRII. mAb 3G8 elicits an increase in $[Ca^{2+}]_i$ derived from intracellular stores through a pertussis toxin insensitive process (24). In parallel, the mAb 3G8-induced actin filament assembly is dependent, in large part, upon an increase in $[Ca^{2+}]_i$. PMN loaded with BAPTA to chelate $[Ca^{2+}]_i$ showed marked inhibition of the response to ligation of $Fc\gamma RIII_{PMN}$ (Fig. 6*A*). This calcium dependence of cytoskeletal rearrangement is similar to that described for adherence induced actin polymerization, in which a rapid rise in $[Ca^{2+}]_i$ may be the signal triggering actin filament assembly (26, 65, 66). In contrast, mAb IV.3 elicits only a minimal change in $[Ca^{2+}]_i$ (24) and mAb IV.3-induced actin polymerization is completely resistant to intracellular calcium chelation at 2 min. This response is more like actin polymerization triggered by FMLP which is calcium independent (Fig. 6*B*) and G protein-dependent (35–37, 67). Of course, the varying role of changes in $[Ca^{2+}]_i$ in the induction of cytoskeletal rearrangement, dependent on the triggering stimulus, indicates that multiple intracellular pathways converge on the regulation of actin filament assembly. The relative persistence of the early response (<15 s) to mAb 3G8 IgG (Fig. 6*A*) and the lack of complete inhibition of the response elicited by 3G8 $F(ab')_2$ and GAM $F(ab')_2$ in BAPTA-treated cells suggest that even a single receptor system may activate several signal transduction pathways. Nonetheless, the differential sensitivity of FcγRII and $Fc\gamma RIII_{PMN}$ to intracellular calcium chelation indicates that different signaling pathways may be preferentially engaged by each receptor. These distinct pathways may underlie differential efficiencies and mechanisms of activation for each of the FcγR in PMN (68, 69).

In addition to mediating certain PMN effector functions, the increase in $[Ca^{2+}]_i$ and filamentous actin generated by $Fc\gamma RIII_{PMN}$ ligation may also serve as a "priming" signal to modify neutrophil responses to other stimuli. Among priming agents, both binding of LPS or platelet activating factor to PMN and adherence by PMN to biologic surfaces lead to increases in both $[Ca^{2+}]_i$ (41, 65, 66, 70) and in F-actin content (26, 42). The effects of adherence, mediated through cross-linking of CD11/CD18 integrins by matrix proteins on the solid phase (41), involve cytoskeletal microfilaments (41) and render receptors for cytokines competent to trigger the respiratory burst (27, 40). Consistent with the notion that $Fc\gamma RIII_{PMN}$ delivers a critical intracellular signal for effector functions, previous data demonstrate the essential role of $Fc\gamma RIII_{PMN}$ in the phagocytosis of Con A-treated rabbit E (20). Similarly, our experiments demonstrate that crosslinking of $Fc\gamma RIII_{PMN}$ with 3G8 $F(ab')_2$ serves to enhance the phagocytic capacity of FcγRII (Fig. 7). Whether other FcγRII functions and other receptors systems are also enhanced awaits further investigation.

The capacity of $Fc\gamma RIII_{PMN}$ to initiate direct effector functions and to prime PMN has important implications for the role of immune complexes in disease. Immune complex deposition may lead not only to FcγRII and FcγRIII initiated release of inflammatory mediators but also to the capacitation or enhancement of the PMN response to other stimuli such as cytokines. Amplification of neutrophil-mediated tissue damage by immune complexes might contribute importantly to the pathogenesis of inflammatory diseases such as SLE and rheumatoid arthritis. Thus, an understanding of the biologic capacities of $Fc\gamma RIII_{PMN}$, and their variation with genetic polymorphisms of the receptor, are essential to the understanding of such immunologic diseases.

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