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Physical Proximity and Functional Interplay of the Glycoprotein Ib-IX-V Complex and the Fc Receptor FcγRIIA on the Platelet Plasma Membrane*

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Paul M. Sullam‡§, William C. Hyun¶, Janos Szöllösi‖, Jing-fei Dong, Wendy M. Foss‡, and José A. López**‡‡**

*From the ‡Department of Medicine, University of California, San Francisco and Veterans Affairs Medical Center, San Francisco, California 94121, the ¶Laboratory for Cell Analysis, Cancer Center, University of California, San Francisco, California 94143, the ‖Department of Biophysics and Cell Biology, Medical University School of Debrecen, Debrecen 4012, Hungary, and the **Departments of Internal Medicine and Molecular and Human Genetics, Baylor College of Medicine and Veterans Affairs Medical Center, Houston, Texas 77030*

Although the glycoprotein (GP) Ib-IX-V complex and FcγRIIA are distinct platelet membrane receptors, previous studies have suggested that these structures may be co-localized. To determine more directly the proximity of GP Ib-IX-V and FcγRIIA, we assessed the effects of anti-GP Ibα monoclonal antibodies on FcγRIIA-mediated platelet aggregation and on the direct binding of polymeric IgG to human platelets. In addition, we directly examined the proximity of FcγRII and GP Ib-IX-V using flow cytometric fluorescence energy transfer and immunoprecipitation studies. Preincubation of platelets with either of two monoclonal antibodies (AN51 or SZ2) directed against GP Ibα completely blocked platelet aggregation by polymeric IgG. Similarly, these antibodies totally inhibited platelet aggregation by two strains of viridans group streptococci known to induce aggregation via FcγRIIA. In addition, AN51 and SZ2 significantly reduced the binding of polymeric IgG to washed fixed platelets. When assessed by flow cytometry, significant levels of bidirectional energy transfer were detected between FcγRIIA and GP Ibα, indicating a physical proximity of less than 10 nm between these receptors. This energy transfer was not due to high receptor density, because no homoassociative energy transfer was seen. Moreover, immunoprecipitation of FcγRIIA from platelet lysates also co-precipitated GP Ibα. These results indicate that GP Ibα and FcγRIIA are co-localized on the platelet membrane and that this association is not random.

The glycoprotein (GP)[1] Ib-IX-V complex and FcγRIIA are distinct platelet membrane receptors for von Willebrand factor and polymeric IgG, respectively. The GP Ib-IX-V complex contains four polypeptides (GP Ibα, GP Ibβ, GP IX, and GP V), which are present in a stoichiometry of 2:2:2:1 on the platelet plasma membrane (1). The precise configuration by which these peptides form a functional receptor is unknown, although more than one copy of each polypeptide per receptor complex is probable (1). This receptor mediates platelet adhesion to sites of blood vessel injury by binding von Willebrand factor in the subendothelium and participates in platelet activation by thrombin by providing a high affinity binding site for this agonist. In contrast to the GP Ib-IX-V complex, the platelet receptor for the Fc portion of IgG, FcγRIIA, is much simpler in structure, consisting of a single transmembrane polypeptide. This receptor is responsible for the aggregation and activation of platelets induced by immune complexes, opsonized bacteria, and certain antibodies that bind other platelet surface proteins (2).

Although GP Ib-IX-V and FcγRIIA are structurally unrelated, a functional interaction between these receptors has been suspected for some time. Moore *et al.* reported that both polymeric IgG and IgG Fc fragments could inhibit platelet aggregation induced by von Willebrand factor and ristocetin (3). The reciprocal phenomenon was also observed, *i.e.* prior incubation of platelets with von Willebrand factor and ristocetin inhibited platelet aggregation by polymeric IgG. More recent studies examining heparin-induced platelet aggregation, which is mediated by anti-heparin antibodies, further suggest a proximity of GP Ib-IX-V and FcγRIIA. In particular, platelet aggregation by heparin can be blocked by antibodies directed against either FcγRIIA or GP Ibα (4, 5). Moreover, anti-GP Ib antibodies inhibit heparin-induced aggregation of platelets obtained from normal donors but not of platelets from individuals with the Bernard-Soulier syndrome, which lack the GP Ib-IX-V complex (4).

Although these findings suggest a physical proximity or functional interaction of FcγRIIA and the GP Ib-IX-V complex, such an association has never been demonstrated directly. In the current studies, we used three distinct approaches to determine whether these receptors are physically associated. First, we assessed whether anti-GP Ibα monoclonal antibodies would affect either FcγRIIA-mediated platelet aggregation (induced by polymeric IgG or antibody-coated bacteria) or the direct binding of polymeric IgG to human platelets. Second, we directly examined the proximity of FcγRIIA and GP Ib-IX-V on the platelet membrane as measured by flow cytometric fluorescence energy transfer. Third, we assessed by co-immunoprecipitation experiments whether these receptors were linked

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§ To whom correspondence should be addressed: Veterans Affairs Medical Center, Division of Infectious Diseases (111W), 4150 Clement St., San Francisco, CA 94121. Tel.: 415-221-4810, ext. 2550; Fax: 415-750-0502; E-mail: sullam@sanfrancisco.va.gov.

‡‡ Established Investigator of the American Heart Association.

[1] The abbreviations used are: GP, glycoprotein; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; PRP, platelet-rich plasma; TSS, Tyrode's salt solution; FET, fluorescence energy transfer; DTSSP, dithiobis(sulfosuccinimidyl propionate.

covalently. As discussed below, these studies strongly indicate that GP Ib-IX-V and FcγRIIA are co-localized on the platelet membrane and that these receptors may be functionally interactive.

EXPERIMENTAL PROCEDURES

Polymeric IgG—Polymeric IgG was produced either by heating or chemically cross-linking an IgG solution. For the former procedure, IgG was purified from human immunoglobulin (Miles Laboratory, Elkhart, IN) by protein A affinity chromatography. IgG aggregates were generated by heating a 2 mg/ml solution of the purified IgG in phosphate-buffered saline at 63 °C for 30 min (6). Macroscopic aggregates were removed by centrifugation (12,000 × *g*, 10 min), and the protein content of the supernatant was determined using a bicinchoninic acid assay (Pierce) (7). To produce chemically cross-linked IgG (8), a 100 mg/ml solution of purified human IgG1 (Sigma) in 0.1 M Tris buffer (pH 8.5) was treated with a 10-fold molar excess of dimethyl suberimidate for 2 h at 30 °C followed by dialysis, centrifugation, and determination of its protein content.

Monoclonal Antibodies—The following monoclonal antibodies (mAb), both unlabeled and conjugated with FITC, were obtained from commercial sources: mAb AN51 (anti-GP Ibα; Immunotech International, Westbrook, ME); mAb SZ2 (anti-GP Ibα; DAKO, Carpinteria, CA); mAb FA6.152 (anti-CD36, Immunotech). mAb WM23 (anti-GP Ibα) was a gift from Dr. Michael Berndt of the Baker Research Institute, Prahran, Victoria, Australia. mAb IV.3 (anti-FcγRII) was purchased from Mederex (West Lebanon, NH) or purified from the ascites of BALB/c mice inoculated with hybridoma IV.3 (American Type Culture Collection, Rockville, MD) using ammonium sulfate precipitation (9) and protein G affinity chromatography as recommended by the manufacturer (Pharmacia Biotech Inc.). Antibodies were conjugated with cyanine 3.18 (Cy3) using the Fluorolink-Ab Cy3 labeling kit as per the manufacturer's instructions (Biological Detection Systems, Pittsburgh, PA). Labeling of the mAbs with FITC or Cy3 had no effect on binding affinity as measured by a previously described competitive assay (10).

Preparation of Human Platelets—Platelet-rich plasma (PRP) and washed platelets were prepared from freshly obtained human blood as described previously (11). To produce PRP, blood was collected in tubes containing 3.8% buffered citrate solution (anticoagulant-to-blood ratio, 1:9) and centrifuged (100 × *g*, 15 min, 25 °C) to remove erythrocytes and leukocytes. PRP was recovered by collecting the uppermost two-thirds of the top layer.

Washed platelets were produced by collecting 5 volumes of blood into a syringe containing 1 volume of acid-citrate-dextrose (85 mM Na citrate, 111 mM glucose, 71 mM citric acid) anticoagulant and prostaglandin I_2 (final concentration, 1 μg/ml; Sigma). The platelets were then washed three times in 140 mM NaCl, 20 mM HEPES, 6 mM glucose, 1 mM EDTA, pH 6.6, and then suspended in Tyrode's salt solution (TSS, Sigma) pH 7.2 (11). For flow cytometry, washed platelets were fixed in 0.8% paraformaldehyde (12), washed, and suspended in TSS. Platelets prepared by this technique were not activated, as shown by minimal expression of surface P-selectin (13), when measured by flow cytometry (data not shown).

Preparation of Bacteria for Aggregometry—*Streptococcus sanguis* strain M99 and *S. salivarius* strain D1 previously have been shown to induce platelet aggregation via FcγRIIA (14). To produce bacterial suspensions for platelet aggregometry, each strain was grown for 18 h at 37 °C in Todd-Hewitt broth and then washed, sonicated, and suspended in TSS as described (11). The concentrations of organisms were determined by counting in a hemacytometer and adjusted to 3×10^9 bacteria/ml by the addition of TSS.

Platelet Aggregometry—The ability of polymeric IgG or bacteria to aggregate platelets was tested by conventional light aggregometry using a single-channel aggregometer (Model 330, Chronolog, Haverton, PA). For polymeric IgG, 50 μl of a 10 mg/ml stock solution was added to 450 μl of washed platelets (3×10^8/ml) suspended in TSS. To assess platelet aggregation by streptococci, washed bacteria suspended in TSS were added to PRP at a final bacterium/platelet ratio of 1:1. Platelet aggregation was analyzed with regard to the time interval between the addition of an agonist to the platelet suspension and the onset of aggregation (lag phase), the rate of aggregation (slope at the midpoint of aggregation), and the maximum change in light transmission. All agonists were tested on multiple occasions using platelets from different donors. Human thrombin (1 unit/ml), ADP (10 μM), or ristocetin (1 mg/ml) served as controls for a positive aggregation response (all reagents from Sigma).

Binding of Polymeric IgG to Human Platelets—The binding of polymeric IgG to human platelets was assessed by flow cytometry. Washed fixed platelets (1×10^8/ml) suspended in TSS containing 1% bovine serum albumin were incubated with 1 mg/ml polymeric IgG for 1 h at 4 °C. After three washings in TSS, the platelets were incubated with 1.5 μg/ml FITC-conjugated goat anti-human $F(ab')_2$ IgG (Caltag, South San Francisco, CA). Platelets were diluted 500-fold, and 10,000 cells/sample were analyzed by flow cytometry using a Becton Dickinson FACScan (San Jose, CA). The threshold for forward scatter was set to eliminate small debris and gated to omit cell doublets and clumps. IgG binding was detected as the mean log FITC fluorescence emission in the Fl1 channel using a 530 ± 30 nm band-pass filter. Platelets incubated with secondary antibody alone served as controls for nonspecific binding. To determine the relative ability of the monoclonal antibodies to inhibit polymeric IgG binding, fixed platelets were incubated for 1 h with 10 μg/ml of mAb IV.3, mAb AN51, or mAb SZ2. After washing twice in TSS, the binding of polymeric IgG was assessed by flow cytometry as described above.

Fluorescence Resonance Energy Transfer—To assess directly the proximity of the FcγRIIA receptor and the GP Ib-IX-V complex on the cell surface, the efficiency of fluorescence energy transfer (FET) between FITC-labeled monoclonal antibodies (donor) and CY3-conjugated monoclonal antibodies (acceptor) was measured by flow cytometry as described previously (10, 15, 16). A FACStar Plus flow cytometer (Becton Dickinson) with dual-laser excitation (488 and 528 nm) was used to determine platelet surface FET measurements. For each sample analyzed, at least 20,000 events were collected. Using threshold settings and light-scatter gating as described above, five intensities were measured on a cell-by-cell basis: (i) forward scattered light at 488 nm; (ii) orthogonal scattered light at 488 nm; (iii) fluorescence emission at 510 ± 15 nm, when excited at 488 nm; (iv) fluorescence emission at >580 nm, when excited at 528 nm; (v) fluorescence emission at >580 nm, when excited at 488 nm (representing energy transfer from FITC to Cy3). The contribution of autofluorescence was determined using unlabeled platelets. To calculate FET efficiency for dual-labeled cells on a cell-by-cell basis, correction factors for spectral overlap were determined from single-labeled platelets.

The calculated transfer efficiency was expressed as the percentage of the excitation energy taken up by the donor (FITC) and transferred to the acceptor (Cy3). This method can accurately detect proximity in the 2–10 nm range (17, 18). Factors that influence efficiency of transfer include the proximity of the donor and acceptor dyes and the concentration of acceptor molecules. To control for the possible influence of a high concentration of acceptor molecules, each pair of epitopes measured was cross-checked by its inverse fluorochrome set (*e.g.* FITC-IV.3/CY3-SZ2 was checked by CY3-IV.3/FITC-SZ2). To better define a specific signal in mAb IV.3-labeled cells, further gating on the list mode data was made to select the brightest 10% of cells. After correction for autofluorescence and spectral overlap, energy transfer efficiencies were calculated as described previously (10, 19).

To confirm that energy transfer between GP Ib-IX-V and FcγRIIA was due to receptor co-localization and not an artifact of high receptor density or coincidental association, we then assessed each epitope bound by the above mAbs for homoassociation (20). Platelets were dual-labeled as described above with (i) FITC-labeled IV.3 paired with Cy3-labeled IV.3, (ii) FITC-labeled AN51 paired with Cy3-labeled AN51, and (iii) FITC-labeled SZ2 paired with Cy3-labeled SZ2. After washing, platelets were analyzed by flow cytometry for evidence of energy transfer.

Immunoprecipitation and Western Blotting—Blood from healthy donors was drawn into acid-citrate-dextrose buffer containing prostaglandin I_2, and PRP was prepared as described above. Immunoprecipitation studies were performed with and without the use of a chemical cross-linking reagent. For the former, 100 μl of PRP were incubated in 2 mmol of dithiobis(sulfosuccinimidylpropionate) (DTSSP, Pierce, Rockford, IL) for 30 min at room temperature, followed by lysis in buffer containing 1% digitonin and a mixture of protease inhibitors (20 mg/ml leupeptin, 1.6 μg/ml benzamidine, 1 mg/ml soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride). For the latter, identical aliquots of PRP were lysed directly without prior treatment with DTSSP.

FcγRIIA was immunoprecipitated from the platelet lysate with mAb IV.3 using fixed *Staphylococcus aureus* cells (Pansorbin beads, Calbiochem). The lysate was first incubated overnight with Pansorbin at 4 °C to remove proteins that bound nonspecifically to the beads. After removing the Pansorbin by centrifugation, mAb IV.3 was added to the lysate to a final concentration of 2.6 μg/ml and incubated for 4 h at 4 °C with rocking. The platelet lysates were then incubated with rabbit anti-mouse IgG secondary antibody (5 μg/ml) (Zymed, San Francisco, CA) for a further 1 h at 4 °C. Pansorbin beads were then added to the

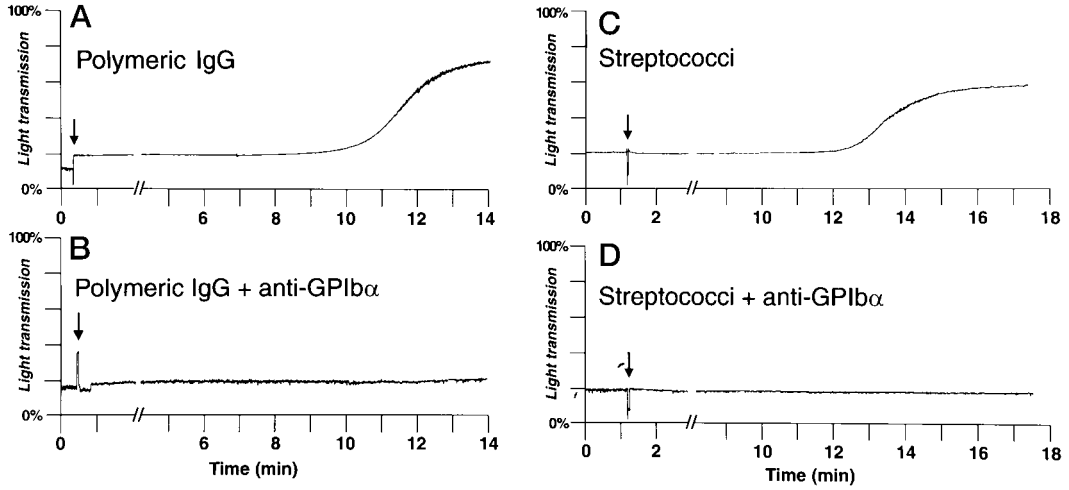

FIG. 1. **Inhibition of FcγRIIA-mediated platelet aggregation by anti-GP Iα mAbs.** *A*, addition of polymeric IgG to washed platelets resulting in a lag phase of 10 min followed by aggregation. *B*, pretreatment of platelets with AN51(10 μg/ml) completely blocked subsequent aggregation by polymeric IgG. *C*, aggregation of platelets by *S. sanguis* strain M99. *D*, complete inhibition by mAb SZ2 (10 μg/ml) of M99-induced platelet aggregation.

lysate and incubated for 1 h at 4 °C. The beads were removed by centrifugation at 10,000 × *g* for 5 min and then resuspended and washed twice in lysis buffer. Bound protein was removed from the beads by boiling in SDS sample buffer containing 2% β-mercaptoethanol.

Solubilized proteins were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Nonspecific protein binding sites on the membrane were blocked by incubation in Tris-buffered saline, pH 7.4, containing 0.2% Tween 20 and 5% nonfat milk for 60 min at room temperature. Co-immunoprecipitated GP Ibα was detected by incubating the membrane with mAb WM23 (0.2 μg/ml) for 60 min at room temperature followed by a further 45 min of incubation with horseradish peroxidase-conjugated sheep anti-mouse IgG. After washing the membrane thoroughly, bound antibody was detected using a commercial chemiluminescence kit (ECL kit, Amersham Corp.) according to the manufacturer's instructions.

Statistical Analysis—Mean values were compared by the unpaired *t* test using analysis of variance and the Bonferroni correction for multiple comparisons.

RESULTS

Effects of mAbs on Platelet Aggregation by Polymeric IgG—Addition of 1 mg/ml of polymeric IgG to washed platelets resulted in a lag phase of 6.5 ± 5.3 min (mean ± S.D.) followed by irreversible platelet aggregation (n = 18) (Fig. 1*A*). As expected, this aggregation was blocked completely by 100 ng/ml mAb IV.3 (6). In addition, platelet aggregation by polymeric IgG was completely inhibited both by mAb AN51 and mAb SZ2, when either mAb was tested at 10 μg/ml (n = 5; Fig. 1*B*). At 1 μg/ml, the effects of these mAbs were variable, with aggregation being either partially inhibited (prolonged lag phase, decreased maximal change in optical transmission) or blocked completely. In control studies, washed platelets treated with SZ2, AN51, or IV.3 aggregated normally in response to thrombin (1 unit/ml), whereas FA6.152 (anti-CD36) had no effect on platelet aggregation induced by polymeric IgG, indicating that inhibition by the above mAbs was relatively specific.

Effects of mAbs on Platelet Aggregation by Viridans Group Streptococci—Platelet aggregation by many strains of viridans group streptococci requires opsonization of bacteria with specific IgG followed by platelet activation via FcγRIIA (11, 14, 21). To confirm that FcγRIIA-mediated platelet aggregation could be inhibited by mAbs to GP Ibα, we examined the effects of mAbs SZ2 and AN51 on platelet aggregation by two strains of viridans group streptococci. Addition of *S. sanguis* strain M99 or *S. salivarius* strain D1 to PRP resulted in mean lag phases of 9.5 ± 2.2 and 11.3 ± 5.1 min, respectively, followed by rapid and irreversible platelet aggregation (n = 5; Fig. 1*C*).

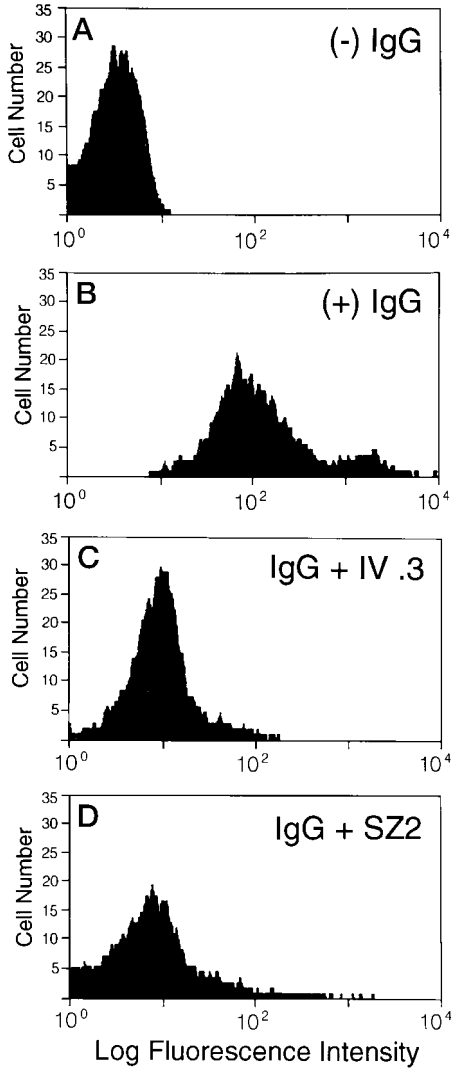

FIG. 2. **Inhibition of polymeric IgG binding to human platelets by anti-GP Iα mAbs.** Binding of polymeric IgG to platelets was measured by flow cytometry using an FITC-conjugated goat anti-human IgG secondary antibody. *A*, platelets alone. *B*, platelets incubated with polymeric IgG. *C*, platelets pretreated with mAb IV.3 prior to incubation with polymeric IgG. *D*, platelets pretreated with mAb SZ2 before incubation with polymeric IgG.

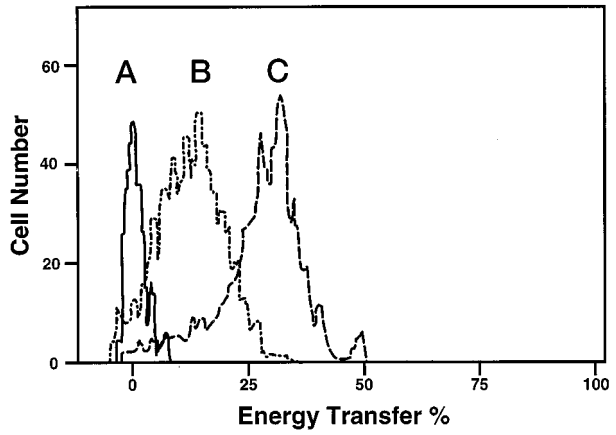

FIG. 3. **Association of FcγRIIA and GP Ibα on the platelet membrane: fluorescence energy transfer analysis.** Energy transfer efficiency was measured between FITC-conjugated and Cy3-conjugated monoclonal antibodies bound to epitopes on the platelet membrane. Results from a representative experiment are displayed as frequency distribution curves. No energy transfer was detected between FITC-IV.3 and Cy3-IV.3 (negative control, *curve A*). Energy transfer was clearly seen between FITC-SZ2 and Cy3-AN51, two non-overlapping epitopes on GP Ibα (positive control, *curve C*). Intermediate levels of energy transfer were observed between FITC-AN51 and Cy3-IV.3 (*curve B*).

As with aggregation induced by polymeric IgG, preincubation of the platelets with mAb IV.3 (100 ng/ml) completely blocked aggregation by these organisms. In addition, platelet aggregation by streptococci was also blocked by 10 μg/ml mAb SZ2 or mAb AN51 ($n = 4$; Fig. 1*D*). Inhibition by these mAbs was selective; ADP normally aggregated platelets pretreated with IV.3, AN51, or SZ2. Moreover, FA6.152 failed to block platelet aggregation by either strain M99 or D1, indicating that the inhibition by mAbs directed against platelet GP Ibα was not merely due to a nonspecific or generalized effect of antibody binding to the platelet surface.

Inhibition of Polymeric IgG Binding to Platelets—We then examined whether mAbs AN51 and SZ2 inhibited aggregation by reducing binding of polymeric IgG to the platelets. As shown in Fig. 2, the binding of polymeric IgG to washed fixed platelets could be readily detected by flow cytometry. When probed with FITC-conjugated goat anti-human $F(ab')_2$, platelets incubated with polymeric IgG appeared as a discrete population clearly separated from platelets treated with secondary antibody alone. Preincubation of platelets with IV.3 reduced binding by 98.8 ± 21.1% (mean ± S.D., $n = 8$, $p < 0.01$), indicating that binding by polymeric IgG to platelets was predominantly mediated by FcγRIIA (Fig. 2*C*). Preincubation of platelets with 10 μg/ml of SZ2 or AN51 also reduced binding significantly, by 98.1 ± 19.8% ($p < 0.01$) and 73.0 ± 19.9% ($p < 0.05$), respectively (Fig. 2*D*; $n = 5$). In control studies, platelets pretreated with anti-CD36 had levels of polymeric IgG binding comparable to those seen with untreated platelets (data not shown).

Analysis of Receptor Co-localization by Fluorescence Energy Transfer—The above results indicated that FcγRIIA and GP Ib-IX-V are in physical proximity on the platelet membrane. To examine more definitively the potential co-localization of these receptors, we measured FET between labeled mAbs directed against epitopes on each receptor (Fig. 3). As expected, the two epitopes on GP Ib-IX-V bound by AN51 and SZ2 showed significant levels of FET (Table I). For example, percent energy transfer from FITC-labeled AN51 to Cy3-labeled SZ2 was 16.3 ± 3.9%. Comparable levels of energy transfer were observed with FITC-labeled SZ2 paired with Cy3-labeled AN51. FET measurements between FcγRIIA and GP Ib-IX-V also indicated significant proximity between these receptors. For example, the mean energy transfer from FITC-labeled AN51 to Cy3-labeled IV.3 was 9.2 ± 3.2% ($p < 0.001$, compared with single-labeled controls). Comparable levels of FET were observed between these epitopes when the donor and acceptor dyes were reversed (FITC-labeled IV.3 and Cy3-labeled AN51). Significant FET was also seen between FITC-labeled IV.3 and Cy3-SZ2 (6.8 ± 3.0) as well as between these epitopes when the fluorochrome labels were reversed (5.2 ± 1.4; $p < 0.01$).

TABLE I
Fluorescence energy transfer

Fluorescence energy transfer efficiency values between platelet membrane epitopes using monoclonal antibodies to glycoprotein Ibα (AN51 and SZ2) and FcγRII (IV.3). Energy transfer was measured between FITC-labeled (donor) and Cy3-labeled (acceptor) epitopes by flow cytometry. $n = 6$ experiments.

Donor (FITC)	Acceptor (Cy3)	Energy transfer (mean ± S.D.)	*p* value
		%	
AN51	SZ2	16.3 ± 3.9	<0.001
SZ2	AN51	23.6 ± 5.7	<0.001
AN51	IV.3	9.2 ± 3.2	<0.001
IV.3	AN51	5.2 ± 2.9	<0.01
SZ2	IV.3	5.2 ± 1.4	<0.01
IV.3	SZ2	6.7 ± 3.0	<0.001
IV.3	IV.3	1.7 ± 0.9	>0.05
AN51	AN51	1.9 ± 1.1	>0.05
SZ2	SZ2	3.7 ± 1.7	>0.05

Energy transfer between two different receptors may be due to true co-localization or may result from a high surface density of receptors, which would result in FET because of crowding on the platelet surface (19). To determine whether the observed FET was due to high receptor density (as opposed to true co-localization), we then examined the epitopes bound by mAbs AN51, SZ2, and IV.3 for homoassociation. No evidence of significant FET was detected for any of the three antibody pairs tested, indicating that neither FcγRIIA nor GP Ibα was expressed at a density sufficient to produce FET via chance transfer.

Physical Association between the GP Ib-IX-V Complex and FcγRIIA—As another means of studying the association of the two receptors, we attempted to co-immunoprecipitate GP Ib-IX-V complex with FcγRIIA using mAb IV.3 and digitonin lysates of platelets. These experiments were performed with or without pretreatment of the platelets with the bifunctional cross-linking agent DTSSP. When tested with lysates of untreated platelets, mAb IV.3 co-precipitated GP Ibα in parallel with FcγRIIA, but with considerable variation in the amount of GP Ibα recovered on repeated testing. However, pretreatment of platelets with DTSSP resulted in the consistent co-precipitation of GP Ibα with FcγRIIA in quantities comparable to those obtained with antibodies against GP V or GP Ibα itself (Fig. 4).

DISCUSSION

Although GP Ib-IX-V and FcγRIIA are seemingly unrelated receptors on the platelet membrane, previous reports had suggested a functional cooperation or structural association of these surface structures. With this study, we provide strong evidence that the functional interaction between GP Ib-IX-V and FcγRIIA is largely due to a direct physical interaction. Several observations support this conclusion. First, we found that mAbs to GP Ibα completely blocked platelet aggregation induced by polymeric IgG, which is an FcγRIIA-mediated process. These antibodies apparently inhibited aggregation by blocking the binding of polymeric IgG to FcγRIIA. Indeed, we

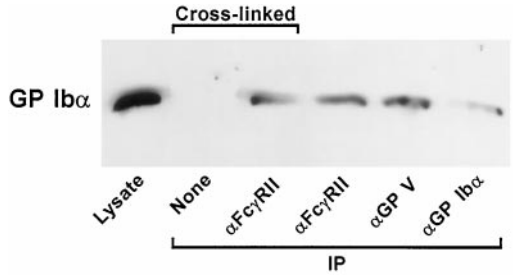

FIG. 4. **Co-immunoprecipitation of GP Iα with FcγRIIA from digitonin-lysed platelets.** Shown is an immunoblot with the anti-GP Ibα mAb WM23. The *left lane* is a control from a lysate of whole platelets. The *subsequent lanes* are immunoprecipitates from the lysates using antibodies against the polypeptides indicated (*αFcγRII*, mAb IV.3; *αGP V*, mAb SW16; *αGP Ibα*, mAb AK2). The lanes containing samples from platelets that had been chemically cross-linked with DTSSP before immunoprecipitation are denoted above the blot.

found that mAbs directed against GP Ibα could significantly reduce platelet binding by polymeric IgG and that these mAbs inhibited binding by amounts comparable with those obtained with the anti-FcγRII mAb IV.3. Because the GP Ibα-specific mAbs do not appear to bind FcγRIIA directly (data not shown), these findings indicate that the above inhibitory activity results from steric hindrance, suggesting that FcγRIIA and GP Ibα are in close proximity on the platelet membrane.

We also demonstrated the proximity of these receptors more directly by two methods, resonance energy transfer between antibodies bound to FcγRIIA and GP Ibα and co-immunoprecipitation experiments. Fluorophor-labeled antibodies bound to the respective antigens were able to transfer emission energy from the FITC-labeled antibody to the Cy3-labeled antibody, regardless of whether the donor dye was associated with FcγRIIA or GP Ibα. The detection of energy transfer between epitopes on FcγRIIA and GP Ibα indicates that these receptors are within 10 nm of each other. This proximity cannot be explained by the random co-localization of FcγRIIA and GP Ibα. Indeed, we observed no homoassociative energy transfer for epitopes on either receptor, indicating that the densities of FcγRIIA and GP Ibα on the platelet membrane are too low to randomly produce FET. Our calculations of receptor density also argue against the association being due to random distribution. Based on previous estimates of platelet surface area (20–80 μm^2) (8) and receptor number (25,000 and 500–8,000 copies/platelet for GP Ibα (1, 17) and FcγRIIA (2, 22), respectively), and assuming random receptor distribution, the predicted distance between these receptors would exceed the limits of detectable FET. Thus, the observed FET in our studies indicates nonrandom association between FcγRIIA and GP Ibα.

We obtained further evidence for a direct physical association between the GP Ib-IX-V complex and FcγRIIA in our platelet in immunoprecipitation studies. Using mAb IV.3, GP Ibα was invariably coprecipitated with FcγRIIA from platelet membranes that had been pretreated with DTSSP, a chemical cross-linking reagent that is membrane-impermeable (23). In the absence of this pretreatment, the GP Ibα was still recovered, but the yield and frequency of recovery varied considerably. These findings indicate that there is a direct interaction between the GP Ib-IX complex and FcγRIIA, although the interaction is weak enough to be disrupted under certain conditions of lysis. Moreover, our ability to chemically cross-link these receptors on the surface of intact platelets provides additional evidence for their proximity.

Of note, platelet aggregation by streptococci was also blocked by mAbs to GP Ibα. In addition to providing corroborative data for the aggregation studies using polymeric IgG, these results may clarify some inconsistent findings on the mechanisms of platelet aggregation by these organisms. Our previous studies had demonstrated that platelet aggregation by many strains was mediated by FcγRIIA (11, 14). A subsequent report, however, indicated that mAbs to GP Ibα could inhibit platelet aggregation by viridans group streptococci, suggesting a role for this receptor (24). Although these divergent observations may reflect different mechanisms of platelet activation used by different species, our current data suggest that the inhibitory activity observed by Ford *et al.* (24) is due to steric blockade of FcγRIIA by the anti-GP Ib antibodies.

What are the potential consequences of the association between the GP Ib-IX-V complex and FcγRIIA? As yet no definitive information exists to answer this question, but one possible reason for their association may be for participation in signal transduction. The interaction between the GP Ib-IX-V complex and von Willebrand factor leads to signal transduction across the platelet membrane, resulting in tyrosine phosphorylation of a number of proteins and an influx of calcium (25–27). As yet, the mechanism for this signal transduction has not been delineated, and in particular it is unclear as to how the GP Ib-IX-V complex is coupled to the signal transduction machinery. Its subunits do not have tyrosine kinase activity, are not known to bind to G-proteins, and are not phosphorylated by tyrosine kinases. Recently, one of the 14–3-3 proteins has been shown to associate with the GP Ib-IX-V complex (28, 29), but as yet no clear mechanism has been proposed for how this association is involved in signal transduction. The association of FcγRIIA with the complex may provide a mechanism for signal transduction by the complex. FcγRIIA is able to transduce activation signals when cross-linked by aggregated IgG or IgG bound to bacteria, as we have demonstrated here. This activation is partly mediated by an interaction of an immune tyrosine-based activation motif (ITAM) in the cytoplasmic domain of FcγRIIA and the SH2 motif containing kinase Syk, a member of the Src family of tyrosine kinases (30, 31). Recent evidence indicates that the GP Ib-IX-V complex must be cross-linked to transmit signals (32), a mechanism similar to that employed by FcγRIIA for signal transmission (31). Thus, if an agonist is able to change the conformation of the GP Ib-IX-V complex such that subcomponents of the complex are brought together, this action may also bring together and cross-link associated FcγRIIA polypeptides, resulting in signal transduction.

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