Platelet Glycoprotein (Gp) IX Associates With Gplba in the Platelet Membrane GpIb Complex

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The platelet membrane glycoprotein (Gp) Ib complex consists of four polypeptides: the disulfide-linked GpIba and GpIbp subunits; GpIX, tightly, but noncovalently associated with GpIba-β and the more weakly associated GpV. It is not certain whether the association of GpIX to GpIba-β is via GpIba, GpIbp, or both subunits, although recently published evidence implicates an interaction with GpIbp. We have investigated the interaction of GpIX with GpIba-β using polyclonal rabbit antibodies to GpIba and GpIbp raised by immunization with purified glycocalcin and with synthetic peptide sequences from GpIbp, respectively, as well as monoclonal antibodies directed against GpIX (FMC-25) and against GpIba (AP-1). We performed two types of experiments, using either purified GpIb complex or platelets. (1) When wells were coated with nonreduced GpIb complex, the binding of FMC-25 was inhibited 73% by the GpIba antibody, but only 30% by the GpIbp antibody; when wells were coated with reduced complex, FMC-25 binding was inhibited by the same two antibodies by 86% and 13%, respectively.

When wells were coated with polyclonal GpIba or GpIbp antibodies and then incubated with reduced GpIb complex, only wells coated with GpIba antibodies captured GpIX reactivity. When wells were coated with FMC-25 and then incubated with nonreduced GpIb complex, both the GpIba and GpIbp polyclonal antibodies reacted strongly; in contrast, only GpIba reactivity was retained when wells coated with FMC-25 were incubated with reduced GpIb complex. In the reciprocal experiment, AP-1-coated wells incubated with either nonreduced or reduced GpIb complex bound radiolabeled FMC-25. (2) The ability of polyclonal GpIba and GpIbp antibodies to inhibit binding of FMC-25 to platelets was studied by ELISA and by flow cytometry. In both systems, FMC-25 binding was inhibited by the GpIba antibody, but not significantly by the GpIbp antibody. We conclude that GpIX is strongly associated with GpIba in the purified platelet GpIb complex and in the platelet membrane.

MATERIALS AND METHODS

Materials

Monoclonal antibodies (MoAbs) to GpIX (FMC-25) and GpIba (AP-1) were generously supplied by Dr Michael Berndt (University of Sydney, Australia), and Dr Robert Montgomery (The Blood Center of Southeastern Wisconsin, Milwaukee, WI), respectively. Sheep antiglycocalcin antiserum was a gift of J. S. N. Tandon and G. Jamieson (The American Red Cross Jerome H. Holland Laboratory, Rockville, MD). Secondary antibodies were obtained from Kirkegaard and Perry Laboratories, Inc (Gaithersburg, MD) and Pierce (Rockford, IL). RIBI adjuvant was purchased from RIBI Immunocore Research, Inc (Hamilton, MT). Sepharose 4B, PD-10 columns, wheat germ agglutinin (WGA) Sepharose 6MB, Protein G Superose and Mono-Q Superose were obtained from Pharmacia Biotech (Piscataway, NJ). Succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) was obtained from Pierce. Keyhole limpet hemocyanin was purchased from Calbiochem (La Jolla, CA). Affigel-10 was purchased from Bio-Rad Laboratories (Hercules, CA). All other reagents were purchased from Sigma Chemical Co. (St Louis, MO). MoAb FMC-25 was radiolabeled with Na131 (ICN Biochemicals, Costa Mesa, CA) using the method of Fraser and Speck.

Preparation of glycocalcin and GpIb complex. Glycocalcin (Gc), the extracellular portion of GpIb, was prepared essentially as described. Fifty-unit batches of 6 to 7 day outdated platelet concentrates (obtained from the American Red Cross, Penn-Jersey Region, Philadelphia, PA) were washed three times in 20 mmol/L Tris-HCl, 0.15 mol/L NaCl, and 10 mmol/L NaEDTA, pH 7.5 (STE), and resuspended in 3 mol/L KCl for 30 minutes at room temperature. The suspension was centrifuged at 3,000 rpm for 15 minutes at room temperature and the supernatant fraction was passed over a 10 mL WGA Sepharose column and eluted with 100 mmol/L N-acetyl-D-glucosamine. Fractions containing Gc were pooled and concentrated by absorption on a Mono-Q 5/5 column and eluted with a 0.1 to 0.7 mol/L NaCl gradient. Gc was separated from lower molecular weight contaminants by gel filtration on a 1.5 × 100 cm Sepharose CL-6B column and concentrated by ultrafiltration. In reduced and nonreduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) the purified Gc consisted of one band at approximately 120 kD. GpIb-IX complex was purified from
washed platelets which were lysed by the addition of an equal volume of 1% Triton X-100 in STE containing 5 U/mL DNase I and protease inhibitors (0.1 mg/mL leupeptin, 400 KIU/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 15 μg/mL pepstatin A). The lysate was applied to the WGA-Sepharose column, washed, eluted with N-acetyl-D-glucosamine (100 mmol/L) in STE with 0.1% Triton X-100, dialyzed against STE and applied to an anti-GpIb MoAb (AP-1) MoAb Sepharose column. After washing with 0.5 mol/L NaCl in the same buffer, the column was eluted with 0.05 mol/L diethanolamine, pH 11.5. Fractions were neutralized with 1 mol/L Tris-HCl. The GpIb complex so prepared contains GpIX but does not contain immunologically detectable amounts of GpV.

Preparation of polyclonal antibodies against GpIX and GpIbp. To prepare antibodies against GpIbp, rabbits were immunized with purified human Gc by subcutaneous injection, using RIBI adjuvant. To prepare GpIbp antibodies, two peptides from the plateau GpIbp chain (residues 81-93 and 101-108) were synthesized and coupled to keyhole limpet hemocyanin, using SMCC as described and mixed with RIBI adjuvant. Rabbits were immunized monthly by subcutaneous injection. Immune sera were obtained after four or five immunizations. Antibodies were detected by immunodiffusion against purified Gc or GpIbp complex and antibody titers were determined by enzyme linked immunosorbent assay (ELISA) in wells coated with either Gc or the GpIbp peptides coupled to bovine serum albumin (BSA). IgG was isolated from immune serum using protein G-Sepharose. The anti-GpIbp IgG was further purified by one of two methods: (1) Total IgG was passed through a keyhole limpet hemocyanin-Affigel-10 column followed by affinity purification on a column of BSA-coupled GpIbp peptides linked to Sepharose-4B; (2) GpIbp peptides were reduced using an immobilized reducing agent (Redeine-1mm, Pierce) and attached to a 2 ml SulfoLink (Pierce) matrix. Total IgG first was passed over a similar column with an irrelevant peptide attached, and the effluent was loaded onto the specific peptide column, which was extensively washed with phosphate buffered saline (PBS). Specifically bound immunoglobulins were eluted with 0.1 mol/L glycine pH 2.7. Fractions were neutralized using 1 mol/L Tris-base. The purified polyclonal antibodies were characterized by Western blotting of reduced (5% β-mercaptoethanol) and nonreduced SDS-polyacrylamide gels. For use as a positive control, polyclonal mouse antihuman platelet antibodies were prepared in our laboratory.

Methods

Direct ELISA. An ELISA assay was used to determine the immunoreactivity of reduced and nonreduced GpIb-IX complex against purified polyclonal GpIbp and GpIbp antibodies. Reduced complex was prepared by incubation of GpIbp complex with 5% β-mercaptoethanol for 30 minutes. Wells were coated with either reduced or nonreduced GpIbp complex (100 μL of a 5 μg/mL solution in 100 mmol/L NaHCO3/Na2CO3, pH 9.6) overnight at 4°C, washed three times with 200 μL PBS (20 mmol/L phosphate, 150 mmol/L NaCl, pH 7.4) containing 0.05% Tween-20 (PBS-T), blocked with 200 μL 3% BSA in PBS-T for 2 hours, washed three times with PBS-T, and incubated for 1 hour with 100 μL polyclonal anti-GpIbp (1 μg/mL) or anti-GpIbp (4 μg/mL). The wells were washed three times with PBS-T, incubated for 1 hour with 100 μL polyclonal anti-GpIbp (1 μg/mL) or rabbit anti-GpIbp (1 μg/mL) or rabbit anti-GpIbp (4 μg/mL). The platelets were washed three times with PBS, incubated with a 1:10 dilution of FITC-conjugated goat antirabbit IgG (Fab′), washed three times with PBS, and analyzed using a Coulter EPICS Profile II fluorescence-activated cell sorter (Coulter Corp., Hialeah, FL). Data are reported as changes in mean log fluorescence intensity.

RESULTS

As shown in Fig 1, on Western blots the anti-GpIbp polyclonal antibody detected a single band of approximately 170 kD or 140 kD using nonreduced or reduced GpIbp complex, respectively, while the polyclonal antisem to GpIbp detected a single band at approximately 170 kD or 24 kD on
nonreduced or reduced GpIb complex, respectively. MoAb FMC-25 detected a single band at 22 kD on nonreduced gels, but reacted poorly on reduced gels. As shown in Table 1, both polyclonal antibodies and FMC-25 reacted strongly against reduced and nonreduced GpIb complex coated on microtiter wells, compared with BSA or pre-immune IgG controls, although absorbance values obtained with reduced complex were slightly lower than those seen with nonreduced complex.

To examine the effect of polyclonal GpIba or GpIbβ antibodies on the binding of MoAb FMC-25 to the GpIb complex, a competitive ELISA was used. As shown in Fig 2, FMC-25 binding to nonreduced GpIb complex was inhibited 73% by the GpIba antibody and only 30% by the GpIbβ antibody, suggesting that GpIX may be more closely associated with GpIba than with GpIbβ. When the GpIb complex was reduced (under conditions that on Western blotting indicated total dissociation of GpIba and GpIbβ) and coated on microtiter wells, anti-GpIba IgG inhibited FMC-25 binding by 86%, while anti-GpIbβ IgG inhibited FMC-25 binding by only 13%, compared with 5% inhibition by normal rabbit IgG.

To investigate further the relationship between GpIba, GpIbβ, and GpIX, we examined the nature of the antigen(s) captured on microtiter wells coated with antibodies to GpIba, GpIbβ, or GpIX. As shown in Fig 3A, the GpIX MoAb FMC-25 reacted strongly with the antigen(s) captured by polyclonal GpIba antibody when either reduced or nonreduced GpIb complex was added, although immunoreactivity was slightly lower when reduced complex was used. As expected, the GpIba MoAb AP-1 and the mouse antihuman platelet antiserum both reacted strongly in this system. Low absorbance values were found when normal mouse IgG (negative control) was substituted for specific antibodies. As shown in Fig 3B, both AP-1 and FMC-25 reacted with the antigen(s) captured by polyclonal GpIbβ antibody when nonreduced GpIb complex had been added. In contrast, neither AP-1 nor FMC-25 reacted with wells coated with polyclonal GpIbβ antibody when reduced GpIb complex was incubated in the well. As expected, mouse antihuman platelet antiserum reacted strongly with reduced and unreduced GpIb complex, while low absorbance values were found when normal mouse IgG was added. In an effort to obtain more direct data concerning the GpIba-GpIX interaction, the GpIba MoAb AP-1 was used to capture either nonreduced or reduced GpIb complex and radiolabeled FMC-25 was added.

![Image](https://www.bloodjournal.org)
INTERACTION OF PLATELET GpIX WITH GpIbα

Fig 3. Antigen capture on microtiter wells coated with anti-GpIbα IgG (A) or anti-GpIbβ IgG (B). See Methods for experimental details. Results are means and ranges (n = 3).

In these experiments similar and substantial amounts of radioactivity bound to the wells when either reduced or nonreduced complex was added (Table 2), again indicating an interaction between the captured GpIbα and the added GpIX, even after reduction. In the converse experiment, as shown in Fig 4, the antigen(s) captured on microtiter wells coated with FMC-25 and incubated with nonreduced GpIb complex reacted with both GpIbα and GpIbβ antibodies. In contrast, only GpIbα antibodies reacted with FMC-25–coated wells incubated with reduced GpIb complex. When BSA was added to microtiter wells in place of GpIb complex no increase in cpm was found. In additional experiments, FMC-25–coated microtiter wells were incubated with nonreduced GpIb complex and we examined the ability of a series of 6 GpIbα MoAbs (50 μg/mL) to inhibit binding of the polyclonal GpIbβ IgG (4 μg/mL). The GpIbα MoAbs inhibited anti-GpIbβ IgG binding by 37% to 67% (data not shown).

Table 2. Radiolabeled FMC-25 Binding to Antigen(s) Captured by MoAb AP-1

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<tr>
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<th>AP-1 Capture</th>
<th>Normal Mouse IgG Capture</th>
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<tr>
<td>Nonreduced complex</td>
<td>1499 ± 531*</td>
<td>105 ± 7</td>
</tr>
<tr>
<td>Reduced complex</td>
<td>1141 ± 127</td>
<td>93 ± 28</td>
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<tr>
<td>BSA</td>
<td>266 ± 48</td>
<td>102 ± 20</td>
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* Mean cpm ± SD (n = 3).

Fig 4. Antigen capture on wells coated with anti-GpIbX MoAb FMC-25. Results are means and ranges (n = 3). Results depicted are for a concentration of GpIbβ antibody of 4 μg/mL; similar results were obtained with a concentration of 2 μg/mL.

These experiments indicate that in the purified GpIb complex GpIX is associated with the GpIbα chain, rather than the GpIbβ subunit. We sought to determine if this relationship held true on the platelet surface. In a series of experiments, platelets coated on microtiter wells were incubated either with FMC-25 alone or with a mixture of FMC-25 and increasing concentrations of GpIbα or GpIbβ polyclonal antibody. As shown in Fig 5, GpIbα IgG inhibited FMC-25 binding to the platelet surface in a concentration-dependent manner. In contrast, anti-GpIbβ IgG had little effect on FMC-25 binding. Since these experiments use fixed platelets, we performed an additional series of experiments using fresh platelets. Gel-filtered platelets were incubated with FMC-25 (2 μg/mL) alone or with mixtures of FMC-25 and either GpIbα or GpIbβ antibodies and bound FMC-25 was detected by flow cytometry. When platelets were incubated with 20, 40, 60, or 120 μg/mL GpIbα antibody, FMC-25 binding was reduced by 30%, 38%, 45%, and 56%, respectively. In contrast, 20, 40, 60, 120, or 240 μg/mL GpIbβ antibody inhibited FMC-25 binding by 0%, 6%, 12%, 14%, and 22%, respectively. These results are identical to those obtained in the ELISA system using fixed platelets.
DISCUSSION

The Gplb complex consists of four polypeptide chains only two of which, GpIba and GpIbβ, are covalently linked. GpIX interacts strongly, but not covalently, with GpIbα-β, but the nature of this interaction has not been established. Recently, Lopez et al, in elegant experiments with transfected cells, reported that GpIbβ was crucial for the synthesis and the surface expression of both GpIX and GpIba. In that report, no evidence of a GpIb-α-GpIX association was found. In this report, we have used both purified Gplb complex and whole platelets to study the interaction of GpIX with GpIbα-β. In both systems we obtained evidence for an association between GpIX and GpIba, and no evidence of an association between GpIX and GpIbβ. The apparent contradiction between our results and those of Lopez et al might have a number of explanations. Several potential problems might arise in studying interactions using the purified Gplb complex. For example, it is conceivable that the steps used to isolate the Gplb complex may have altered, in some manner, one of the subunits of the complex and, therefore, the interactions of the component polypeptides. Although we cannot completely rule out such a possibility, direct examination of whole platelet lysates, prepared either with Triton X-100 or digitonin, gave results identical with those we have observed with the isolated complex (data not shown). A second possibility might be that removal of the detergent, which occurs as part of the purification of the complex, might have contributed to artifactual results in the ELISA assays. However, we have examined the platelet lysates in the presence of 1% digitonin—a condition in which the reactivities of the antibodies we used are unaffected—and obtained results identical with those seen with purified complex. A third possible problem is that all three of the antibodies we used, particularly the polyclonal anti-Gplbβ, reacted somewhat less well against reduced than against non-reduced complex. Nevertheless, it seems unlikely that this small loss in sensitivity could account for a total loss of our ability to detect complex formation between GpIbα-β and GpIX. Finally, the nature of ELISA assays, which involve multiple wash steps, might result in failure to detect weak interactions between GpIbα-β and GpIX. None of these considerations would explain our finding of a strong interaction between GpIX and GpIba.

Nevertheless, studies of isolated membrane complexes cannot be as informative as studies of the complex in situ. We therefore measured the ability of our antibodies to compete for antigen on the platelet surface, using ELISA (with fixed platelets) and flow cytometry (using unfixed platelets). The monoclonal antibodies to GpIbα and GpIbβ, as well as the monoclonal antibodies to GpIba and GpIX, each bind well to the platelet surface. In the whole platelet systems our results lead to the same conclusions we reached using purified Gplb complex: GpIX appears to interact with GpIbα, but not GpIbβ.

Lopez et al previously demonstrated that CHO cells express GpIbα efficiently on the plasma membrane only if triply transfected with cDNAs for GpIbα and GpIbβ and GpIX. Their more recent experiments, using CHO and L cells transfected with only two of the three cDNAs, suggest that GpIbβ is essential for efficient expression of GpIbα and GpIX. The direct evidence for a GpIbβ-GpIX association was from a series of immunoprecipitation experiments. In cells cotransfected with GpIbβ and GpIX, antibodies to either component precipitated both. On the other hand, in cells cotransfected with GpIbα and GpIX, neither a GpIbα MoAb nor a GpIX MoAb coprecipated the other molecule of the complex. However, in those experiments FMC-25 (the GpIbα MoAb) also failed to precipitate GpIX, suggesting that cells cotransfected with GpIbα and GpIX express very little GpIX. The inefficient expression of GpIX in these cells may account for the inability to detect an interaction between GpIbα and GpIX, perhaps even when these cells were transiently transfected with GpIbβ. Finally, as clearly stated in the report by Lopez et al, the results they obtained were largely representative of the intracellular, rather than membrane, pool of the three polypeptide chains and, therefore, might be a function of intracellular conditions, such as the heterogeneity in glycosylation which they observed. Nevertheless, the work of Lopez et al makes clear that the GpIbβ subunit plays a very significant role in directing the translation and processing of the Gplb complex.

The present study examines the interactions between the components of the Gplb membrane complex isolated from normal human platelets, and between the same components within the platelet membrane. Our results indicate a close association between GpIX and GpIba, rather than GpIbβ, and are consistent with a previous report of a 35% inhibition of FMC-25 binding to platelets by affinity-purified rabbit anti-GpIbα IgG. Taken together, our results and those of Lopez et al suggest that GpIX may interact with both GpIbα and GpIbβ, possibly at different times during the expression of the complex. Further studies are needed in order to define more precisely the interactions of these polypeptides.

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