The Human Platelet Membrane Glycoprotein Complex GP IIb-IIIa Expresses Antigenic Sites Not Exposed on the Dissociated Glycoproteins

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A number of recent reports have described murine monoclonal antibodies that react specifically with the complex formed by human platelet membrane glycoproteins (GP) IIb and IIIa. We show that the IgG L, a previously described human alloantibody isolated from a polytransfused thrombosthenia patient, has similar properties. When used in nonprecipitating amounts in crossed immunoelctrophoresis (CIE), 125I-IgG L bound strongly to the IIb-IIIa complex. However, after dissociation of the complex with EDTA, only a weak binding to GP IIb and no binding to GP IIIa was detected. In further studies, increased amounts of IgG L were interacted with 125I-labeled membrane glycoproteins in (a) CIE and (b) classical indirect immunoprecipitation experiments. Although the antibody was able to quantitatively precipitate the IIb-IIIa complex from Triton X-100-soluble extracts of platelet membranes, no precipitation of GP IIb or GP IIIa was observed after divalent cation chelation. Addition of EDTA to immunoprecipitates containing GP IIb-IIIa resulted in dissociation and partial release of both glycoproteins. The interaction of the IgG L with electrophoretically separated GP IIb and GP IIIa was studied using a Western blot procedure in the presence of Ca2+, Mg2+, or EDTA. The presence of divalent cations did not increase the reactivity of the antibody with the individual glycoproteins. Overall, our results show that acquired antibodies to IIb-IIIa, such as the IgG L, may predominantly react with complex-dependent determinants.

The IgG L refers to an IgG alloantibody that was located in the serum of a polytransfused patient with Glanzmann's thrombosthenia. Platelet function tests performed in the presence of the antibody showed that it was a potent inhibitor of aggregation of normal human platelets induced by adenosine diphosphate (ADP), epinephrine, collagen, and thrombin. Subsequent studies showed that the IgG L inhibited the specific binding of 125I-fibrinogen to ADP-stimulated platelets. These and other studies (reviewed by Nurden and Caen) all suggested that the platelet antigen recognized by the antibody plays a central role in a late stage of the mechanism of platelet aggregation.

It was therefore important to identify the platelet antigen recognized by IgG L. Using an indirect immunoprecipitation procedure, Degos et al. found that the antibody reacted with an uncharacterized 125I-labeled membrane constituent of apparent molecular weight 120,000 daltons. Subsequently, it was shown by both Hagen et al. and Kunicki et al. that the IgG L formed an immunoprecipitate containing both glycoproteins (GP)IIb and IIIa when the antibody was used in crossed immunoelctrophoresis (CIE) together with Triton X-100 (BDH Chemicals Ltd, Poole, England)—soluble platelet extracts. It is now known that GP IIb and GP IIIa are present as divalent cation-mediated heterodimer complexes (GP IIb-IIIa) in detergent-soluble platelet extracts, such as were used in the above studies.

A number of authors have reported murine monoclonal antibodies that react with GP IIb-IIIa and inhibit platelet aggregation induced by physiologic agonists. In particular, two antibodies, AP-29 and T10, have been shown to react with epitopes expressed by GP IIb-IIIa, but which are not present on the individual glycoproteins dissociated by EDTA. Binding studies with the same antibodies suggested that the GP IIb-IIIa complex was present in the unactivated platelet membrane. In view of these studies, we reexamined the interaction of IgG L with GP IIb-IIIa. Classical indirect immunoprecipitation procedures have been used together with CIE to investigate whether or not the IgG L exclusively precipitated GP IIb-IIIa, or whether it also interacted with the individual glycoproteins dissociated from the complex by divalent cation chelation. Further experiments were performed to assess the interaction between the IgG L and electrophoretically separated GP IIb and GP IIIa renatured by incubation with nonionic detergent and Ca2+ or Mg2+ prior to the addition of the antibody. Results suggest that IgG L contains antibodies of different specificities, but that the bulk are against determinants present only when GP IIb and GP IIIa are associated as the heterodimer complex.

MATERIALS AND METHODS

Rabbit anti-human whole serum and rabbit anti-human IgG were obtained from Nordic Immunology Labs (Tilberg, The Nether-
lands). Serum from a patient with posttransfusion purpura, known to contain antibody against the platelet alloantigen PI\textsuperscript{a}, was kindly given to us by Dr Bernadette Boizard (Hôpital Lariboisière, Paris). Protein A-Sepharose CL4B and Ficol 400 were purchased from Pharmacia Fine Chemicals (Upplands, Sweden). Materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), high and low molecular weight protein standards, Biogel P2, enzyme-biorad, and nitrocellulose membrane were from Bio-Rad (Richmond, Calif); Triton X-100; Berol EMU-043 from Modokemi (Stenungsund, Sweden); agarose-type HSA from Litex (Glostrup, Denmark); and lactoperoxidase, leupeptin (synthetic), Nonidet P-40, and bovine serum albumin (BSA) (fraction V) from Sigma Chemical Co (St Louis). Carrier-free Na\textsuperscript{251} (100 mCi/mL) in NaOH solution, pH 8–11 and \textsuperscript{125}I-protein A (specific activity 9 \mu Ci/\mu g protein) were from New England Nuclear (Dreieich, West Germany). Autoradiography was performed using Kodak X-Omat \textsuperscript{f2L}' film (Kodak-Pathé, Paris), Cronex cassettes (18 x 24 cm), and Cronex Lightning Plus intensifying screens (Dupont de Nemours, Orsay, France).

**Platelet Isolation**

Unless otherwise stated blood (10 vol) was taken by venepuncture into polypropylene tubes containing 0.134 mol/L EDTA (0.3 vol). The blood was centrifuged at 120 g for 15 minutes at 15 \degree C to prepare platelet-rich plasma. Platelets were routinely washed three times in 10 mmol/L Tris-HCl, 0.15 mol/L NaCl, 1 mmol/L EDTA, pH 7.4 (Tris-EDTA), containing 5 mmol/L glucose as described by Nurden et al. 14

**Preparation of Platelet Membranes**

Platelets were isolated by the above procedures from 1 to 3 units of acid citrate dextrose (ACD) anticoagulated blood obtained from blood bank donors. On occasion, aliquots (1 mL) of washed platelets in Tris-EDTA at 10\textsuperscript{8} platelets/mL\textsuperscript{−1} were taken for lactoperoxidase-catalyzed \textsuperscript{125}I-labeling of their surface proteins in the presence of 1 mCi Na\textsuperscript{125}I. 14 The radiolabeled platelets were diluted 20 to 25-fold with nonlabeled platelets and washed twice more in Tris-EDTA. The procedure used for membrane isolation was given by Pidard et al. 16 Briefly, platelets were centrifuged through a 0% to 40% glycerol gradient and osmotically lysed according to the method of Barber and Jamieson. 17 After their isolation over a 30% (wt/wt) sucrose and osmotically lysed according to the method of Barber and Jamieson. 17 After their isolation over a 30% (wt/wt) sucrose gradient, the membranes were washed twice with 10 mmol/L Tris-HCl, 0.25 mol/L sucrose, pH 7.4 (Tris-sucrose). In some experiments, the membrane preparations were divided into two samples after the first wash and resuspended in either (1) Tris-sucrose or (2) Tris-sucrose containing 5 mmol/L EDTA. The suspensions were agitated at 4 \degree C for 30 minutes prior to the sedimentation of the membranes by centrifugation at 100,000 g for one hour at 4 \degree C. The membranes in each sample were then washed once more in Tris-sucrose prior to their solubilization with Triton X-100.

**Purification and \textsuperscript{125}I-Labeling of Human IgG**

IgG was isolated from the serum of the polyspecific rabbit antimouse IgG (anti-human IgG, and by affinity chromatography on protein A-Sepharose CL4B. 14 Eluted fractions were characterized by immunoelectrophoresis against rabbit anti-human serum, or rabbit anti-human IgG, and by SDS-PAGE. Routinely, the IgG was dialyzed against 0.038 mol/L Tris, 0.1 mol/L glycine, pH 8.7 (Tris-glycine) containing 0.02% wt/vol sodium azide, and concentrated to approximately 2 mg/mL over an Amicon UM 10 filter. Prior to radio labeling, samples of the purified IgG were dialyzed against three changes of 0.1 mol/L sodium phosphate, pH 7.4, for 24 hours at 4 \degree C. Then, 50 \mu L of IgG was mixed with 50 \mu L of rehydrated Enzymobeads, 5 \mu L Na\textsuperscript{125}I (0.5 mCi), 100 \mu L 0.1 mol/L sodium phosphate, pH 7.4, and 50 \mu L 1% (wt/vol) \beta-D-glucose. Radio labeling was then performed exactly as described by Kunicki et al. 14 The specific activity of the \textsuperscript{125}I-labeled IgG was normally in the range 20 to 30 mCi/mg. \textsuperscript{125}I IgG preparations were stored at 4 \degree C in Tris-glycine in the presence of 0.02% sodium azide.

**Crossed Immuno-electrophoresis**

**Sample preparation.** Washed platelets were resuspended at 5 \times 10\textsuperscript{8} platelets/mL\textsuperscript{−1} and isolated membranes at 1 to 2 mg protein/mL\textsuperscript{−1} in Tris-glycine. A one-tenth vol of 10% (vol/vol) Triton X-100 in Tris-glycine was then added, the mixture agitated at 4 \degree C for 30 minutes, and insoluble material pelleted by centrifugation at 100,000 g for one hour at 4 \degree C. In some experiments, final concentrations of 1% Nonidet P-40 or 1% Berol EMU-043 were substituted for the Triton X-100. Protein concentration in the detergent-soluble extracts was determined by the method of Markwell et al. 18

**Electrophoresis.** Routinely, samples of Triton X-100–soluble extracts of whole platelets (100 \mu g protein) or isolated membranes (10 \mu g protein) were electrophoresed at 10 V/cm for one hour in first-dimension gels consisting of 1% (wt/vol) agarose and 0.5% (vol/vol) Triton X-100 in Tris-glycine. 18 Second-dimension electrophoresis was performed at 2 V/cm for 18 hours into the biphasic gel system described by Kunicki et al. 14 This consisted of (1) an intermediate gel containing 1% agarose, 0.5% Triton X-100 and, on occasion, trace amounts of radiolabeled IgG L or precipitating amounts of the same antibody, and (2) an upper gel similarly composed of agarose and Triton X-100 but containing precipitating concentrations of the IgG fraction of a polyspecific rabbit anti-human platelet antiserum. All procedures were performed at 15 \degree C. Details of the antibody concentrations and of any variations to the standard procedure are to be found in the text and figure legends. The preparation of rabbit antiserum against washed human platelets and the isolation of rabbit IgG by ammonium sulphate precipitation and DEAE-cellulose chromatography were as previously described. 14 Antibody pools 1, 4, and 5 were used in this study. As emphasized by Kunicki et al. 14 some variation in the standard pattern of immunoprecipitates may be observed with different antibody pools. Immunoprecipitates were located by Coomassie blue staining. 3 Those that contained \textsuperscript{125}I-labeled antigens were identified by autoradiography. Dried gels were placed against Kodak X-Omat MA film and left for seven days at room temperature. Films were developed according to the manufacturer's instructions.

**Dissociation of Triton X-100–Solubilized GP IIb-IIIa Complexes by EDTA**

In initial studies, the procedures of Kunicki et al. 14 were followed and whole platelet extracts in Triton X-100 were incubated with 5 mmol/L EDTA at 4 \degree C for periods ranging from one hour to 24 hours. However, even after 24 hours, a complete dissociation of GP IIb and GP IIIa was rarely obtained. In subsequent experiments, conditions for optimal dissociation of GP IIb-IIIa were reevaluated using Triton X-100 extracts of isolated membranes at 2 mg protein/mL\textsuperscript{−1}. Among the parameters tested were pH and temperature. To test the effect of pH, identical membrane preparations were suspended in Tris-glycine, pH 8.7, and Tris-sucrose, pH 7.4. Disassociation was performed at 4 \degree C or 22 \degree C. In some experiments, leupeptin (100 \mu g/mL) and phenylmethylsulfonyl fluoride (PMSF) (1 mmol/L) were added as an additional precaution against protease activity. Neither compound affected GP IIb-IIIa dissociation. As shown in Table 1, complete dissociation was achieved by solubilizing membranes with 1% Triton X-100 in...
Table 1. Dissociation of the GP IIb/IIIa Complex in Triton X-100 Using EDTA

<table>
<thead>
<tr>
<th>Membrane Extraction Buffer</th>
<th>1 h at 4°C</th>
<th>2 h at 4°C</th>
<th>24 h at 4°C</th>
<th>1 h at 22°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-sucrose, pH 7.4</td>
<td>(+)</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Tris-glycine, pH 8.7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
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Membranes were prepared from 125I-labeled platelets and solubilized with 1% Triton X-100 in the above buffers, as described in Materials and Methods. Samples of soluble protein (2 mg/mL) were incubated in the presence of 5 mmol/L EDTA as indicated. gpIIb/IIIa dissociation was estimated following OIE; both Coomassie blue-stained gels and autoradiographs were examined. The above results are typical of those obtained for three different membrane preparations: (+) — little or no dissociation; + — less than 25% of complex dissociated; ++ — less than 50% dissociation; +++ (+) — 75% to complete dissociation; ++++ complete dissociation. ND, not determined.

Indirect Immunoprecipitation

Membranes were isolated from 125I-labeled platelets, and soluble extracts in Triton X-100 incubated in the presence or absence of EDTA, as described above. Different amounts of soluble protein (see Results) in a constant volume of 100 μL were reacted with 25 μL of a 2 mg/mL solution of purified IgG L or nonimmune IgG for one hour at 37°C. Then, a tenfold excess of the isolated immunoglobulins of a rabbit anti-human IgG antiserum was added and the incubation continued for 48 hours at 4°C. The precipitates were sedimented by centrifugation at 12,000 g for two minutes in an Eppendorf centrifuge and washed three times in 10 mmol/L Tris-HCl, pH 7.4, containing, in successive washings, 0.5, 0.25, and 0.15 mol/L NaCl. The total radioactivity in the final washed pellets was measured in a Beckman 7,000 gamma counter (Beckman Instruments, Gagny, France). Results were expressed as a percentage of the total radioactivity in the sample or as a percentage of the total protein-bound radioactivity in the sample. The latter was estimated by the addition of cold 10% (wt/vol) trichloroacetic acid to the Triton X-100-soluble platelet membrane extracts. After incubation for one hour at room temperature, the precipitated material was sedimented by centrifugation at 12,000 g for one minute. The pellets were washed in 10% trichloroacetic acid, and the 125I present measured in the gamma counter. In a typical experiment, 62% of the radioactivity present in a 1% Triton X-100 extract of platelet membranes was precipitated by the trichloroacetic acid.

SDS-PAGE

Washed platelets suspended at 2 × 10⁹ platelets/mL in 10 mmol/L Tris-HCl, 0.15 mol/L NaCl, 3 mmol/L EDTA, 5 mmol/L N-ethylmaleimide, pH 7.0, were solubilized by heating at 100°C for five minutes after the addition of a one-fifth vol of 12% (wt/vol) SDS. Isolated membranes (2 mg protein/mL) or individual immunoprecipitates were treated similarly. When performed, disulfides were reduced by incubation with 5% (vol/vol) 2-mercaptoethanol for one hour at 37°C. SDS-PAGE was performed using 7% to 12% gradient acrylamide slab gels according to the procedures described by Nurden et al. When immunoprecipitates were analyzed, the quantity of protein deposited onto the gel was varied according to the amount of radioactivity in the sample. Usually, 200 μg protein was considered a maximum; amounts in excess resulted in abnormal band patterns due to an overloading of the IgG-related polypeptides. After electrophoresis, proteins were detected in either of two ways. In the first method, gels were frozen at −20°C during one hour, then individual sample lanes were sliced horizontally into 1-mm slices using a Bio-Rad Model 190 gel slicer. The radioactivity in each slice was measured in a gamma counter. Alternatively, the gels were dried onto filter paper and the 125I-labeled proteins detected by autoradiography at −80°C using Kodak X-Omat MA film and two intensifying screens.

Western Blotting

Nonreduced samples (50 μg protein) of SDS-solubilized platelets were electrophoresed on 7% to 12% gradient acrylamide slab gels as outlined above. The methods used for the transfer of the separated polypeptides from unstained gels to nitrocellulose membrane and for the rest of the immunoblot procedure were similar to those detailed by Kieffer et al. Prior to contact with the antibody, each membrane strip was routinely soaked for three hours in 50 mmol/L Tris-HCl, pH 8.1, containing 0.15 mol/L NaCl, 0.15 mol/L CaCl₂, 0.04% (wt/vol) Ficoll, 1.5% (wt/vol) bovine serum albumin, 0.04% (wt/vol) sodium azide, and 0.1% (vol/vol) Nonidet P-40. On occasion, the 0.15 mol/L CaCl₂ was replaced by 0.15 mol/L MgCl₂ or 5 mmol/L EDTA; in the latter case, isotonicity was maintained by increasing the concentration of NaCl. Strips were incubated for two hours in the presence of different dilutions (see text) of (1) serum from the patient L, (2) serum containing anti-PVw antibody, or (3) nonimmune serum. After washing, each strip was then incubated overnight with 1 mL (300,000 cpm) 125I-protein A in buffer. The strips were again washed, dried, and the radiolabeled bands detected by autoradiography.

RESULTS

Crossed Immunoelectrophoresis in the Presence of 125I-Labeled IgG L

In the experiments illustrated in Fig 1, a partial dissociation of GP IIb-IIIa was induced by incubating Triton X-100–soluble platelet extracts at 4°C for two hours in the presence of 5 mmol/L EDTA. The immunoprecipitates containing GP IIb-IIIa, dissociated GP IIb, and dissociated GP IIIa have been identified previously and are labeled on the figure. In Fig 1, the intermediate gel contained trace amounts of 125I-IgG L, the aim being to detect those antigens able to bind the antibody. In confirmation of previous studies, in the absence of divalent cation chelation, the 125I-IgG L bound exclusively to the GP IIb-IIIa complex (Fig 1C). In contrast, dissociated GP IIb bound only small amounts of the antibody and GP IIIa none at all (Fig 1D). The apparent affinity of the radiolabeled antibody for the remaining complex was maintained after sample incubation with EDTA. When the IgG from nonimmune human serum was radiolabeled and used under identical conditions, no binding to GP IIb-IIIa was observed (not illustrated).

Crossed Immunoelectrophoresis Using 125I-Labeled Membrane Glycoproteins in the Presence of Increased Amounts of IgG L

The results illustrated in Fig 1 do not exclude the presence of a subpopulation of GP IIb or GP IIIa, still...
ALLOANTIBODY TO PLATELET GP IIb/IIIa

Fig 1. Use of CIE to study the binding of $^{125}$I-IgG L to GP IIb-IIIa and its dissociated components. Washed platelets were solubilized in 1% Triton X-100 in Tris-glycine, pH 8.7, and samples of the soluble proteins incubated at 4 ºC for two hours in the absence (A, C) or presence (B, D) of 5 mmol/L EDTA. Samples (100 µg protein) were electrophoresed (left to right) in first-dimension agarose gels containing 0.5% Triton X-100, and then in the second dimension against a rabbit antiplatelet antibody preparation (pool 1) (750 µg/cm²) in the upper gel and $^{125}$I-labeled IgG L (13 µg/cm²) in the intermediate gel. The gels were washed, dried, and the precipitates located by Coomassie blue staining (A, B). Those antigens that had bound $^{125}$I-IgG L were revealed by autoradiography (C, D).

Studies on the Solubilization of the GP IIb-IIIa Complex by Nonionic Detergent

Crucial to the interpretation of our results is proof that GP IIb-IIIa complex formation is not induced as a result of the experimental conditions employed. When two other nonionic detergents, Nonidet P-40 or Berol EMU-043, were substituted for Triton X-100 during platelet membrane solubilization and during CIE, the precipitate given by IIb-IIIa remained the major arc on the CIE pattern. In other experiments, Triton X-100 was added to membrane suspensions at final concentrations of 1%, 0.5%, 0.1%, and 0.05% (vol/vol), respectively, prior to protein solubilization at 4 ºC during 30 minutes. Even at a final concentration of 0.05% Triton X-100, approximately 50% of the total membrane protein was solubilized. The Triton X-100-soluble fractions were analyzed by CIE; in these experiments, the amount of detergent in the agarose was adjusted to that of the sample being analyzed. At each Triton X-100 concentration, the IIb-IIIa precipitate predominated on the stained gel (not illustrated). No evidence was obtained to suggest the presence of noncomplexed IIb or IIIa at the lower Triton X-100 concentrations.

Fig 2. CIE performed using $^{125}$I-labeled membrane glycoproteins and increased amounts of IgG L in the intermediate gel. Membranes isolated from $^{125}$I-labeled platelets were incubated for 30 minutes at 4 ºC in the presence of 5 mmol/L EDTA. After washing, the membranes were solubilized with 1% Triton X-100 in the absence of chelating agent. Samples (10 µg) were electrophoresed in first-dimension gels containing 0.5% Triton X-100, and then in the second dimension against a rabbit antiplatelet antibody preparation (pool 4) (750 µg/cm²) in the upper gel and either (A) buffer or (B) IgG L (1.3 mg/cm²) in the intermediate gel. The $^{125}$I-labeled antigens were detected by autoradiography of dried, Coomassie blue-stained gels. Typical autoradiographs are illustrated.

Classical Indirect Immunoprecipitation Experiments

In these studies we used Triton X-100 extracts of membranes isolated from $^{125}$I-labeled platelets. Dissociation of GP IIb-IIIa complexes was performed using 5 mmol/L EDTA under the optimal conditions defined in Table 1. Initial experiments involved incubating different concentrations of untreated $^{125}$I-labeled pro-
teins in a fixed volume with a standard amount of IgG L. After one hour at 37 °C, a tenfold excess of IgG from a rabbit anti-human IgG antiserum was added.

Figure 3A shows that the IgG L precipitated [125]labeled antigen(s) in a concentration-dependent manner until there was an apparent antigen excess. In Fig 3B, the results are expressed as a percentage of the total protein-bound radioactivity. In contrast, the Triton X-100-soluble membrane extracts. When total trichloroacetic acid-precipitable radioactivity in

The experiments illustrated in Figs 1 through 3 suggest that the bulk of the antibodies that constitute the IgG L are “complex-dependent” (see Discussion). If this was so, we reasoned that addition of EDTA to preformed immunoprecipitates, such as that labeled P1 in Fig 3A, would result in dissociation and a loss of antibody-binding capacity of GP IIb and GP IIIa. Figure 5 confirms that EDTA did indeed induce the release of bound radioactivity, with up to 50% being released into the supernatant over a period of one hour at 37 °C. SDS-PAGE analysis of the supernatant fractions followed by autoradiography revealed the presence of GP IIb and GP IIIa; densitometric scanning showed that they were present in the same relative amounts as in the original immunoprecipitate (not

![Fig 3. Classical indirect immunoprecipitation experiments performed using IgG L. Membranes isolated from [125]I-labeled platelets were solubilized with 1% Triton X-100 in Tris-glycine buffer, pH 8.7, and aliquots of the soluble extracts incubated for one hour at 22 °C in the presence or absence of 5 mmol/L EDTA. Samples containing different amounts of protein were then incubated with IgG L, or nonimmune IgG, followed by addition of a rabbit anti-human IgG antibody preparation, as detailed in Materials and Methods. The radioactivity in the washed precipitates was measured in a gamma counter. Illustrated are results for a typical experiment in which Triton X-100-soluble membrane extracts were reacted with IgG L (1) without divalent cation chelation (O—O); (2) after divalent cation chelation (A—A); or (3) with nonimmune IgG without divalent cation chelation (O—O). In A, results are expressed as the total radioactivity (cpm) in the precipitate, and in B as the percentage of the total protein-bound radioactivity in the sample (see Materials and Methods). Here, the membrane protein concentration (abscissa) has been plotted on an arbitrary scale. P1 and P2 refer to the immunoprecipitates analyzed in Fig 4.

![Fig 4. Analysis by SDS-PAGE of the [125]I-labeled proteins present in immunoprecipitates obtained using IgG L. Precipitates P1 and P2 (Fig 3A) were solubilized in SDS and 200 μg protein from each applied to 7% to 12% acrylamide gradient slab gels without prior reduction of disulfides. Following electrophoresis, the individual lanes of protein-stained gels were cut into 1-mm slices. The distribution of [125]I in the gel slices was then quantitatively determined. The relative migration of protein standards of known molecular weight is marked (1).

![Fig 5. Release by EDTA of bound [125]I-radioactivity from immunoprecipitates obtained using IgG L. Identical precipitates to P1 in Fig 3A were resuspended in Tris-glycine, pH 8.7, and incubated at 37 °C either in the absence (B) or presence (E) of 5 mmol/L EDTA. After 0.1, or 10 hours, duplicate samples were centrifuged at 12,000 g for two minutes in an Eppendorf centrifuge. The amount of radioactivity present in the supernatant is expressed as a percentage of the total radioactivity in the sample. A typical experiment is illustrated.](https://www.bloodjournal.org/content/1250/1/1150)

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ALLOANTIBODY TO PLATELET GP IIb/IIIa

illustrated). EDTA did not induce solubilization of the IgG L-anti-human IgG complexes.

**Antibody Binding in the Presence of Divalent Cations to Electrophoretically Separated GP IIb and GP IIIa**

A Western blot procedure was used to further study the interaction between IgG L and separated GP IIb and GP IIIa. Platelet proteins separated by SDS-PAGE were electrophoretically transferred to strips of nitrocellulose membrane. After transfer, the strips were incubated with buffer containing Nonidet P-40 and CaCl₂, MgCl₂, or EDTA. Antibody binding to the reconstituted antigens was then studied, with bound antibody located using 125I-protein A and autoradiography. The presence of antibodies able to react directly with GP IIb was confirmed (Fig 6). However, these were detected only at low serum dilutions (1:2, 1:4), and their reactivity was much less than was located using an anti-Pl antibody reacting with GP IIIa under identical conditions and at a 1:10 dilution (Fig 6). Pretreatment of the separated antigens with calcium, or magnesium (not illustrated), did not increase binding of the IgG L. Control experiments performed with nonimmune IgG or 125I-protein A alone were negative.

**DISCUSSION**

The development of antibodies to platelet-specific antigens is a potential hazard of blood transfusion. In posttransfusion purpura (PTP), antibody production in otherwise normal individuals is a consequence of a different phenotype expression of platelet alloantigens on donor, as compared to recipient, platelets.\(^2\)\(^1\)\(^2\)\(^2\) Although a recent study has shown the presence of antibodies reacting with GP IIb in one patient,\(^2\)\(^5\) most characterized antibodies in PTP are directed against the Pl\(^\text{IIa}\) platelet alloantigen\(^2\)\(^1\) carried by GP IIIa.\(^2\)\(^3\) A special category of posttransfusion antibodies may occur in patients with inherited deficiencies of platelet membrane glycoproteins, such as in Glanzmann’s thrombasthenia and the Bernard-Soulier syndrome (see Nurden\(^2\)\(^4\) for a review). Multiple transfusions to arrest bleeding episodes in these patients run the risk of provoking antibody production against the missing glycoproteins. Such antibody production has been described.\(^1\)\(^2\)\(^5\)\(^2\)\(^6\)\(^2\)\(^6\) The IgG L is an alloantibody isolated from the serum of a polytransfused patient with Glanzmann’s thrombasthenia. The present study was designed to define as clearly as possible the antibody specificities that constitute the IgG L.

Our results confirmed those of Hagen et al\(^5\) and Kunicki et al\(^6\) by showing that the alloantibody is directed against determinants present on the GP IIb-IIIa complex. Quite striking, however, was the difference between the strong reaction between the IgG L and IIb-IIIa complexes and the weak reactivity observed in experiments performed after divalent cation chelation. Apart from the antibodies directed to the complex, CIE showed the presence of some antibodies capable of binding to dissociated GP IIb, and this was confirmed using a Western blot technique and glycoproteins separated by SDS-PAGE. The failure of the anti-GP IIb antibodies to precipitate dissociated GP IIb in the immunoprecipitation experiments may reflect a different reactivity of IgG L to GP IIb in the various immunologic procedures employed. Alternatively, the effectiveness of CIE, indirect immunoprecipitation, and immunoblot procedures in detecting antibodies reacting with noncomplexed GP IIb may represent a reflection of the relative sensitivities of the different procedures in identifying what appears to be a minor subpopulation of antibodies. It is well known that the Western blot technique is a highly sensitive method for studying antigen–antibody interactions.\(^2\)\(^7\)

Although it is now recognized that GP IIb-IIIa complex formation is mediated by divalent cations,\(^6\)\(^8\)\(^2\)\(^8\) little is known of the mechanism whereby the glycoproteins are attached one to another. Crucial to any indirect immunoprecipitation experiment with free GP IIb and GP IIIa is the ability to achieve and maintain a complete dissociation of GP IIb-IIIa complexes in nonionic detergent. Incubation of Triton X-100–soluble membrane extracts at pH 8.7 with EDTA at 4 °C resulted in a slow and inconsistent

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**Fig 6.** Western blot showing the interaction between electrophoretically separated GP IIb and GP IIIa with IgG L in the presence or absence of Ca\(^2\)\(^+\). Samples (50 μg) of nonreduced SDS-soluble platelet proteins were separated by SDS-PAGE prior to their transfer to nitrocellulose membrane (see Materials and Methods). Individual strips were incubated with buffer containing Nonidet P-40 and either CaCl₂ (a) or EDTA (b,c) prior to their interaction with serum from the patient I (a, b) or with anti-Pl \(^\text{IIa}\) antiserum (c) diluted 1:2 (IgG L) or 1:10 (anti-Pl\(^\text{IIa}\)) with buffer. Bound IgG was located with 125I-protein A. The nitrocellulose strip (d) was not incubated with antiserum or with protein A, but contained proteins transferred following the electrophoresis of samples solubilized from 125I-labeled platelets.
were difficult to design. This was possible using the interaction of the IIb-Illa complex. Thus, experiments to dissociate GP IIb and GP IIIa results in the reconstitution of GP IIb and GP IIIa, but in the absence of EDTA in the sample (Fig 2), suggested that EDTA itself was not blocking the interaction between the antibody and its determinants. At least three explanations can be put forward to explain our findings: (1) the IgG L may bind to determinants whose structure is partially given by each glycoprotein and that are only present when GP IIb and GP IIIa are complexed together; (2) the association of GP IIb with GP IIIa may result in a changed conformation of one or both glycoproteins, with new antigenic sites expressed distant to these involved in complex formation; or (3) the antibody binding occurs to sites that are themselves divalent cation-dependent and are altered when EDTA is added to bring about complex dissociation.

As shown by several authors, simple addition of Ca\(^{2+}\) or Mg\(^{2+}\)-containing solutions to mixtures of dissociated GP IIb and GP IIIa results in the reconstitution of the IIb-IIIa complex. Thus, experiments to investigate the interaction of the IgG L with the individual glycoproteins in the presence of divalent cations were difficult to design. This was possible using the immunoblot procedure because the electrophoretically separated GP IIb and GP IIIa were no longer in contact, thus, complex formation could not occur. However, a similar weak binding of IgG to GP IIb was observed in the presence of EDTA, MgCl\(_2\), or CaCl\(_2\), while no binding to GP IIIa occurred under any conditions. That binding of IgG to GP IIIa could occur under the conditions used was confirmed by a positive reaction of an anti-Pl\(^{A1}\) serum with this glycoprotein. Although an initial, irreversible inhibition by SDS cannot be entirely excluded, our results do not favor the possibility that IgG L binds to determinants that are themselves divalent cation-dependent and do not directly require complex formation.

Overall, our findings suggest that IgG L contains antibodies that react with GP IIb, but that the bulk of the IgG react with antigenic sites that are lost when IIb and IIIa are dissociated from the heterodimer. It is already apparent from studies with murine monoclonal antibodies that GP IIb-IIIa is highly immunogenic when human platelets or isolated membranes are used as the antigen source. Our studies, albeit with an extreme example—that of a polytransfused thrombocytopenia patient—suggest that complex-specific determinants of GP IIb-IIIa are potentially important contributors to antibody production in immune platelet disorders. The recent demonstration of autoantibodies against GP IIb-IIIa in some patients with chronic idiopathic thrombocytopenic purpura emphasizes this possibility.

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The human platelet membrane glycoprotein complex GP IIb-IIIa expresses antigenic sites not exposed on the dissociated glycoproteins

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