Further Studies on the Interaction Between Human Platelet Membrane Glycoproteins IIb and IIIa in Triton X-100

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Analysis of human platelet membrane proteins by crossed immunoelectrophoresis (CIE) in the presence of Triton X-100 (TX-100) has previously shown that glycoproteins (GP) IIb and IIIa are located in a single immunoprecipitate, band 16. To investigate whether IIb and IIIa are associated in a complex, we have analyzed TX-100-solubilized labeled membrane proteins by density gradient ultracentrifugation using 10%-40% sucrose gradients containing the nonionic detergent. Studies were performed using soluble proteins derived from membranes isolated in the presence or absence of EDTA. Analysis of gradient fractions by SDS-polyacrylamide gel electrophoresis showed that in the absence of divalent cation chelation, GP IIb and IIIa penetrated well into the gradient (fractions 15–17). Analysis of fractions 15–17 by CIE revealed the presence of band 16. In contrast, when the membrane proteins were incubated with EDTA prior to or after TX-100 solubilization, IIb and IIIa remained near the top of the gradient (fractions 8–11) and gave separate immunoprecipitates during CIE. Incubation of washed platelet lysates with leupeptin, an inhibitor of the Ca2+-dependent protease of human platelets, had no effect on the shape of the band 16 immunoprecipitate. Thus, for the first time, direct evidence has been obtained that GP IIb and IIIa may form a divergent cation-mediated complex. Calibration of the sedimentation profiles using proteins of known molecular weight suggests that the complex is of limited size. Indirect evidence suggests that the complex is a heterodimer.

Several reports have suggested that two platelet membrane glycoproteins, GP IIb and IIIa, may be present as a complex following membrane solubilization by nonionic detergents. The first such observation was made by Hagen et al., who analyzed Triton X-100 (TX-100) solubilized proteins from whole platelets by crossed immunoelectrophoresis (CIE) using a polyspecific rabbit anti-human platelet antibody preparation. Using platelets whose surface proteins had been labeled with 125I by the lactoperoxidase procedure, Hagen et al. observed the presence of a prominent, asymmetrical immunoprecipitate that was heavily radiolabeled. This precipitate was termed band 16. On analysis of isolated membranes, band 16 was sometimes markedly reduced in size. When this was so, two new 125I-labeled precipitates showing lines of identity with the major precipitate were noted. Excision of band 16 from unstained agarose gels enabled the analysis of the contained proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography. The presence of both GP IIb and IIIa in band 16 was revealed. Shulman and Karpatkin also observed a major immunoprecipitate (termed band 10) on analysis of TX-100-solubilized platelet membrane proteins by CIE. Band 10 appeared to be the same immunoprecipitate as that termed band 16 by Hagen et al., as it was severely decreased in platelets of patients with Glanzmann’s thrombasthenia, an observation first made by Hagen et al. Shulman and Karpatkin noted at least two other precipitates with apparent lines of identity with what they referred to as the “major antigen.”

Using a different approach, McEver et al. showed that a monoclonal hybridoma antibody that was directed against a platelet membrane antigen, retained both GP IIb and IIIa when TX-100-solubilized membranes were passed over an affinity column prepared using the antibody. McEver et al. interpreted their results as showing that the monoclonal antibody reacted with an antigen carried by GP IIb and/or IIIa and that these polypeptides were subunits of a major platelet membrane glycoprotein complex.

The above observations were clarified by Kunicki et al., who directly precipitated band 16 during CIE using a human alloantibody (IgG . . . L). SDS-PAGE analysis of both anodal and cathodal sections of the immunoprecipitate revealed the presence of GP IIb and IIIa in equal proportions throughout the length of the precipitation arc. At the same time, these authors showed that when washed platelets were solubilized with Triton X-100 in the presence of EDTA or EGTA, band 16 was severely reduced in size and was replaced by two new precipitates containing either IIb or IIIa alone. This effect of EDTA could be reversed by the subsequent addition of excess calcium. Finally, Kunicki et al. showed that a dissociation of IIb and IIIa occurred when isolated platelet plasma membranes were washed with buffer containing EDTA.
even if the solubilization was performed in the absence of further additions of chelating agent.

In the present study we have used density gradient ultracentrifugation to investigate the presence of glycoprotein complexes following the solubilization of isolated platelet membranes with TX-100. Membranes incubated in the presence or absence of EDTA have been examined. Analysis of the gradient fractions after centrifugation of 125I-labeled membrane proteins has allowed the detection of the major radiolabeled components of the platelet surface. In this way we have been able to confirm that the interaction between IIb and IIIa in TX-100 represents a specific association and that a major effect of EDTA on the proteins of isolated membranes is the dissociation of this complex.

MATERIALS AND METHODS

Platelet Isolation

In a typical experiment, platelets were isolated by differential centrifugation from 4 U of fresh acid-citrate-dextrose (ACD)-anticoagulated blood obtained from healthy, adult donors. The platelets were washed 3 times in 0.01 M Tris-HCl, 0.15 M NaCl, 0.001 M EDTA, pH 7.4 (Tris-EDTA) before being resuspended in Tris-EDTA at a final concentration of 10^9 platelets/ml. Three milliliters of this suspension were taken for lactoperoxidase-glycerol gradient and osmotically lysed according to the method of preferential addition of chelating agent.

Preparation of Platelet Membranes and TX-100-Solubilized Proteins

The 125I-labeled platelets were centrifuged through a 0%–40% glycerol gradient and osmotically lysed according to the method of Barber and Jamieson. EDTA was included in all buffers used in procedures after the sedimentation of the glycerol-loaded platelets unless otherwise mentioned. After isolation over a 30% (w/w) sucrose cushion, the membrane-enriched fraction was diluted with 0.01 M Tris-HCl, 0.25 M sucrose, pH 7.4 (Tris-sucrose) and pelleted by centrifugation at 100,000 g for 1 hr at 4°C. The membranes were washed twice with Tris-sucrose and divided into 2 equal fractions. The first of these was resuspended in Tris-sucrose containing 5 mM EDTA, pH 7.4. The suspensions were incubated at 4°C for 30 min, after which the membranes were sedimented by centrifugation at 100,000 g for 1 hr at 4°C. The pellets were each resuspended in 0.5 ml 0.038 M Tris-HCl, 0.1 M glycine, pH 8.6 (Tris-glycine) and TX-100 added to a final concentration of 1% (v/v). Solubilization was performed with agitation at 4°C for 30 min. Non-solubilized material was pelleted as described above using the SW 50.1 rotor of a Beckman L-3-50 ultracentrifuge (Beckman Instruments, Gagny, France). The rotor being fitted with adaptors for 0.8 ml, 5 x 42 mm cellulose nitrate tubes. Protein concentrations in the presence of TX-100 were determined by the method of Markwell et al. Bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, Mo.) was used as the standard.

Sucrose Density Gradient Ultracentrifugation

Identical linear 10%–40% sucrose density gradients were prepared using an ISCO (model 570) gradient former (Instrumentation Specialties Company, Lincoln, Neb.). Sucrose gradients were prepared in 10 mM Tris-HCl, pH 8.6, with or without 5 mM EDTA. TX-100 was maintained at 1% throughout each gradient. The volume of the gradients was approximately 4.5 ml. In a typical experiment, 0.23 ml sample volumes containing 300–500 µg TX-100-solubilized membrane proteins, with a specific radioactivity (125I) of 2–3 x 10^6 cpm/mg protein, were deposited onto each gradient. Samples solubilized from membranes that had not been treated with EDTA were applied to gradients that did not contain EDTA. Each gradient was centrifuged for 16 hr at 145,000 g max. in the SW 50.1 rotor of a Beckman L-3-50 ultracentrifuge. All operations were performed at 4°C. Following centrifugation, the gradients were fractionated into 100-µl fractions using an ISCO (model 183) density gradient fractionator. In this procedure the tubes were pierced at the base and the sucrose solutions displaced with a high density liquid (Fluorinert FC-48, ISCO). An aliquot from each fraction, normally 40 µl, was immediately mixed with a one-fourth volume of 0.01 M Tris-HCl, 5 mM N-ethylmaleimide, 3 mM EDTA, 10% (w/v) SDS, pH 7.0. After 10 min at 37°C, the samples were frozen and stored at -20°C until SDS-PAGE was performed. The rest of each fraction was used for radioactivity counting, protein determination, and when performed, analysis of the contained proteins by CIE. An identical gradient, but with buffer added instead of protein samples, was always centrifuged in parallel with the gradients containing the TX-100-solubilized proteins. Following the fractionation of this gradient, the refractive index of each 100-µl sample was determined using an Abbé 60 Refractometer (Bellingham and Stanley Ltd., Tunbridge Wells, England).

Verification of the Gradients Using Protein Standards

The following purified proteins were used: human myeloma IgM (mol wt 900,000) (a gift of the department of Prof. Seligmann, Hôpital Saint-Louis, Paris, France), human fibrinogen (mol wt 340,000) (a gift of Dr. J. Soria, Hôpital Hotel Dieu, Paris, France), polyclonal human IgG (mol wt 147,000) (Hoechst-Behring, Paris, France), human transferrin (mol wt 76,000) (Serva, Heidelberg, W. Germany), and soybean trypsin inhibitor (mol wt 21,500) (Sigma). The proteins were dissolved at 5 mg/ml in 10 mM Tris-HCl, 0.15 M NaCl, pH 8.6, containing 1% v/v TX-100. A mixture (200 µl) containing 200 µg of each protein was applied to a 10%-40% sucrose gradient (without EDTA) and ultracentrifugation performed in parallel with that of a sample of TX-100-soluble 125I-labeled membrane proteins (without EDTA treatment). After centrifugation and fractionation of the gradients as described in the previous section, a 25-µl sample from each fraction was analyzed by SDS-PAGE using 7%–12% gradient slab gels (see below). The proteins were located by Coomassie blue R250 (CBB) staining. IgM was detected by analysis of the gradient fractions by fused rocket immunoelectrophoresis using a rabbit antiserum directed against the Fc fragment of human IgM (Nordic Immunological Laboratories, Tilburg, Holland).

SDS-PAGE Procedures

Single dimension SDS-PAGE was performed using 7.0%–12.0% gradient acrylamide slab gels of dimensions 14 x 10 x 0.15 cm. A 3% acrylamide stacking gel was employed, and the sample wells were made using a 20-well template. The electrophoresis system was that of Laemmli;11 the procedures used have been detailed elsewhere.12 Disulfide reduction was performed by incubating the SDS-solubilized proteins from each gradient fraction for 1 hr at 37°C in the presence of 5% (v/v) 2-mercaptoethanol. Electrophoresis was
performed for 18–20 hr at 25 V (constant voltage) in a Model 220 Vertical Slab Gel Apparatus (Bio-Rad Laboratories, Richmond, Calif.). A sample (80 μg protein) of TX-100-solubilized membranes was also electrophoresed on each gel in parallel with the gradient fractions. Proteins were detected by CBR-staining and 125I-labeled proteins identified by autoradiography of the dried gels against Kodak X-Omat MA film as described by Nurden et al.12 Molecular weight determinations were made relative to the migration of reduced protein standards electrophoresed on the same gel. The following standard proteins were used: myosin heavy chain (mol wt 200,000), β-galactosidase (mol wt 116,250), phosphorylase-B (mol wt 92,500), bovine serum albumin (mol wt 66,200), ovalbumin (mol wt 45,000), carbonic anhydrase (mol wt 31,000), and soybean trypsin inhibitor (mol wt 21,500). These proteins were obtained from Bio-Rad as part of the high and low molecular weight standard protein kits.

Crossed Immunoelectrophoresis

TX-100-solubilized platelet membrane proteins and some selected fractions taken from the sucrose density gradients were analyzed by CIE using a polyspecific rabbit anti-human platelet antibody preparation; methods were as detailed by Hagen et al.1,2 and by Kunicki et al.3 Polyspecific antibodies were obtained from 10 rabbits immunized against washed human platelets according to the immunization procedure of Bjerrum and Bøgg Hansen.13 Sera taken over a 3-mo period were pooled and the IgG isolated by ammonium sulfate precipitation and DEAE-cellulose chromatography.3 In the present set of experiments, a pool of antibodies prepared from sera obtained 12–15 mo after the primary immunization was used. TX-100-solubilized platelet membrane proteins (15–20 μg), or samples (20 μl) taken from the sucrose density gradients, were electrophoresed at 10 V/cm for approximately 1 hr in first-dimension gels consisting of 1% agarose, 0.5% (v/v) TX-100 in Tris-glycine. Second-dimension electrophoresis was performed for 18 hr at 2 V/cm into a biphasic gel system that consisted of (1) an intermediate gel containing 1% agarose, 0.5% TX-100 in Tris-glycine and (2) a superior gel containing 1% agarose, 0.5% TX-100 in Tris-glycine and the rabbit anti-platelet immunoglobulin preparation (750 μg/cm). Temperature was maintained at 15°C throughout the electrophoresis. Proteins were located by CBR-staining, and precipitates containing 125I-labeled proteins were detected by autoradiography.5 Protein concentrations of the isolated immunoglobulin preparation were calculated from the absorbance at 280 nm using an extinction coefficient of ε280 = 15.0 for rabbit IgG.

Inhibition of Ca2+-Dependent Protease Activity

Washed platelet suspensions in Tris-glycine were solubilized with 1% TX-100 as described by Kunicki et al.3 Solubilization was performed in the presence or absence of 500 μg/ml leupeptin (crystalline, Sigma). This concentration of leupeptin has been shown to prevent hydrolysis of platelet actin-binding protein in platelet lysates.14 SDS-PAGE analysis of the TX-100-soluble protein confirmed the inhibition of Ca2+-dependent protease activity by leupeptin (results not shown).

RESULTS

Solubilization of Human Platelet Membrane Proteins Using TX-100

Membrane-enriched fractions were prepared from 125I-labeled platelets using the glycerol lysis procedure. When the membranes were stirred with 1% TX-100 at pH 8.6 during 30 min at 4°C, approximately 80% of the total membrane protein was solubilized. SDS-
PAGE analysis of the soluble and insoluble fractions was performed. Results showed that most of the membrane proteins had been solubilized, these included the $^{125}$I-labeled glycoproteins as evidenced by autoradiography following the electrophoresis. The SDS-PAGE patterns were similar to those that we have presented elsewhere when isolated membranes were solubilized with 0.5% TX-100 in 0.01 M Tris-HCl, pH 7.0. The major components of the 100,000 g sedimentable material were a prominent band of mol wt 200,000 and a mol wt 46,000 protein that is thought to represent membrane-associated actin. Incubation of the washed membranes with 5 mM EDTA itself resulted in the solubilization of <5% of the total membrane protein. SDS-PAGE analysis of this fraction revealed several faint bands but no clear evidence of the preferential solubilization of specific membrane proteins. Prior incubation with EDTA had no effect on the subsequent solubilization of the $^{125}$I-labeled glycoproteins by TX-100.

Fig. 2. Sedimentation profile of the individual CBR-staining membrane proteins. Membranes were prepared from $^{125}$I-labeled platelets and incubated with or without EDTA, as described in the legend to Fig. 1. TX-100 solubilization and density gradient ultracentrifugation were performed, and a sample volume (20 μl) from each 100-μl fraction was incubated with SDS and treated with 2-mercaptoethanol as described in Materials and Methods. The samples were electrophoresed on 7%–12% polyacrylamide slab gels using the Laemmli buffer systems. Proteins were located by CBR staining. The sedimentation profile of CBR-stained proteins solubilized from membranes incubated with 5 mM EDTA (A) is compared with that of CBR-stained proteins solubilized from membranes untreated with EDTA (B). P, protein standards detailed in Materials and Methods; M, a sample (80 μg) of the TX-100-solubilized protein that was applied to the sucrose gradient.
Density Gradient Ultracentrifugation of the 125I-Labeled Membrane Glycoproteins

It is now well documented that when the surface proteins of washed human platelets are labeled with 125I using the lactoperoxidase-catalyzed procedure much of the radioactivity is incorporated into membrane GP IIIa.6,19 Thus, by first radiolabeling the surface proteins, we were able to follow the sedimentation of complexes containing GP IIIa when TX-100-solubilized membrane proteins were analyzed by ultracentrifugation on 10%-40% sucrose gradients containing the nonionic detergent. Figure 1 illustrates the distribution of radioactivity along the gradient after the centrifugation of 125I-labeled proteins solubilized from membranes that had either been incubated with 5 mM EDTA, or isolated in the absence of contact with divalent cation chelating agents. In the absence of EDTA, two major peaks of radioactivity were detected encompassing fractions 8–13 and fractions 14–20, respectively, with the highest cpm being found in fractions 14–20. In contrast, after divalent cation chelation, a single major peak of radioactivity remained near the top of the gradient in fractions 8–13. It appeared that incubation with EDTA had modified the sedimentation profile of the major 125I-labeled membrane glycoproteins.

Analysis of the Sucrose Gradient Fractions for CBR-Staining Proteins

In order to identify the membrane constituents responsible for the shift in the 125I-profile, the proteins contained within each fraction of gradients identical to those illustrated in Fig. 1 were analyzed by SDS-PAGE using 7%-12% gradient acrylamide slab gels. Typical CBR-stained gels are illustrated in Fig. 2A and B. The distribution along the gradient of proteins solubilized from membranes that had been incubated with EDTA is shown in Fig. 2A. The fractions 5–11, containing the peak of radioactivity, were enriched in two bands of mol wt 118,000 and 100,000 (Table 1). When the same samples were electrophoresed in the absence of disulfide reduction (results not shown), the mol wt of the bands changed to 134,000 and 89,000, respectively. Such clearly distinctive changes in electrophoretic mobility confirmed that the bands represented the major polypeptide chains of GP IIb and IIIa (see Ref. 5, 6, and Table 1). These bands became progressively weaker in fractions 11–17. No trace of GP IIb or IIIa and no new CBR-stained bands were observed on analysis of fractions 21–40. Figure 2B shows the protein distribution in the gradient following centrifugation of proteins solubilized from membranes that had not been incubated with EDTA after platelet lysis. GP IIb and IIIa were now predominantly located in fractions 11–16. This was a specific displacement as indicated by CBR-staining (compare Fig. 2A and 2B). Attention is also drawn to the fact that in the gradient illustrated in Fig. 2B, the relative staining intensities of GP IIb and IIIa within each fraction are similar. In contrast, following divalent cation chelation (Fig. 2A) there is no such symmetry and IIb has tended to penetrate further into the gradient than IIIa.

Membrane proteins of apparent mol wt 200,000, 46,000, 30,000, and 22,000 were prominent on both gels. However, we did note that in the continued presence of EDTA, these proteins tended to aggregate and sediment towards the base of the tube. This accounts for the lower CBR staining of these proteins in the gradient fractions analyzed in Fig. 2A. The major CBR-staining polypeptide with an apparent mol wt 46,000 represents that fraction of membrane-associated actin that is solubilized by nonionic detergents,17 while the mol wt 200,000 polypeptide probably represents myosin heavy chain.

Analysis of the Sucrose Gradient Fractions for 125I-Labeled Glycoproteins

CBR staining allowed the detection of GP IIb and IIIa. A number of other membrane glycoproteins that are labeled with 125I and that may contribute to the radioactivity in the profiles shown in Fig. 1 stain poorly with CBR.6,19 In order to locate the additional glycoproteins, fractions from identical gradients to those illustrated in Figs. 1 and 2 were analyzed by SDS-
Fig. 3. Sedimentation profile of the individual $^{125}$I-labeled membrane glycoproteins. Membranes were prepared from $^{125}$I-labeled platelets and incubated with or without EDTA as described in the legend to Fig. 1. TX-100 solubilization and density gradient ultracentrifugation were performed and a sample volume (20 $\mu$l) from each fraction of the gradient analyzed by SDS-PAGE, as illustrated in Fig. 2. $^{125}$I-labeled proteins were located by autoradiography of dried CBB-stained gels. The sedimentation profile of $^{125}$I-labeled glycoproteins solubilized from membranes incubated with 5 mM EDTA (A) is compared with that of $^{125}$I-labeled proteins solubilized from untreated membranes. M, a sample (80 $\mu$g) of the TX-100-solubilized protein that was applied to the sucrose gradient. The arabic numerals to the right of Fig. 3A designate the bands identified in Table 1. Two artefactual bands are to be noted in the lower molecular weight region of fraction 17 (B); these were not observed on analysis of the same fraction from other identical gradients.
PAGE and the $^{125}$I-labeled proteins revealed by autoradiography. Results are shown in Fig. 3. Autoradiography confirmed the markedly reduced penetration of GP IIb and IIIa into the gradient following EDTA treatment. In addition, other radiolabeled bands could now be distinguished. In order to identify the membrane glycoproteins giving rise to these bands, further studies were performed. Samples from each gradient fraction were analyzed by SDS-PAGE without disulfide reduction (experiment not illustrated). The apparent molecular weight of each band was then determined reduced and nonreduced. The results are presented in Table 1. Identification of the other bands was also aided when samples of $^{125}$I-labeled Bernard-Soulier platelets, lacking GP Ib, and $^{125}$I-labeled platelets of a patient with Glanzmann's thrombasthenia, lacking GP IIb and IIIa, were electrophoresed under identical conditions (results not shown). Based on the accumulated data, band 1 on Fig. 3A was identified as that termed GP Ia by Phillips and Poh Agin. This glycoprotein penetrated well into the gradient despite the presence of EDTA. Band 2 in Fig. 3A contained GP IIa as the major $^{125}$I-labeled component. This band was primarily located in fractions 8-11 after incubation with EDTA but appeared to have penetrated further into the gradient in the absence of divalent cation chelation. The presence of glycoprotein complexes other than between IIb and IIIa may be indicated by these observations.

**Analysis of the Sucrose Gradient Fractions by CIE**

Samples taken from fractions 9 and 16 of the gradients whose analysis is illustrated in Fig. 3 were also examined by CIE using the immunoglobulin fraction of a rabbit antiserum prepared against normal human platelets. Aliquots of the TX-100-soluble membrane proteins that were applied to the sucrose density gradients were first analyzed (results not shown). The precipitation patterns obtained for the $^{125}$I-labeled proteins were identical to those reported by Kunicki et al.
IIb and al. Thus, as identified by these authors, in the absence of divalent cation chelation, the bulk of GP IIb and IIIa were located in the same precipitate, while following EDTA treatment, IIb and IIIa were located separately in different precipitates. CIE of fraction 9 of gradient B, Fig. 3 (membranes not treated with EDTA) revealed the presence of a small amount of free GP IIIa and only a trace of the purported IIb/IIIa complex (Fig. 4A). Analysis of fraction 16 of the same gradient (Fig. 4B) revealed the presence of a prominent $^{125}$I-labeled precipitate in the position of the IIb/IIIa complex. In contrast, when fraction 9 of gradient A, Fig. 3 (membranes incubated with EDTA) was examined, prominent precipitates corresponding to free GP IIb and free GP IIIa were present (Fig. 4C). Analysis of fraction 16 of the same gradient (Fig. 4D) revealed that these precipitates were only present in small amounts in this fraction. No trace of the precipitate given by the purported IIb/IIIa complex was located. As an aid to the identification of the different precipitates, attention is drawn to the increased migration of the IIb/IIIa complex in the first dimension (2.0–2.5 cm) compared to the migration of free GP IIIa (approximately 1.5 cm).

Examination of Fig. 4 (B and D) shows that fraction 16 of both gradients contained an additional $^{125}$I-labeled precipitate with a first-dimension migration intermediate between that of free GP IIIa and the IIb/IIIa complex. Reference to Fig. 3A, in particular, strongly suggests that this precipitate is being given by GP Ia, a result consistent with our previous study when different immunoprecipitates were excised from unstained plates and the contained antigens identified by SDS-PAGE.

Inhibition of the Ca$^{2+}$-Dependent Protease

In order to verify that changes in the migration of IIb and IIIa during CIE were not a result of Ca$^{2+}$-dependent protease activity in the absence of EDTA, platelets were solubilized with TX-100 in the presence of leupeptin, a potent inhibitor of the enzyme. No modifications in the shape of the IIb/IIIa immunoprecipitate were observed.

Attempt to Define the Approximate Size of the IIb/IIIa Complex

Membranes were again isolated from $^{125}$I-labeled platelets and the $^{125}$I-labeled proteins solubilized using TX-100. No divalent cation chelation was performed. A sample of the solubilized material was applied to a 10%–40% sucrose gradient and ultracentrifugation performed as described in Figs. 1–4. A mixture of proteins of known molecular weight (see Materials and Methods) was applied to an identical gradient that was centrifuged in parallel to that containing the $^{125}$I-labeled membrane proteins. Following centrifugation, the gradients were fractionated and the sedimentation of the protein standards analyzed by SDS-PAGE or fused rocket immunoelectrophoresis. Only IgM (mol wt 900,000) penetrated into the lower half of the gradient (highest concentrations in fractions 30–35). Fibrinogen (mol wt 340,000) was most concentrated in fractions 18–22 and penetrated into the gradient just in front of the IIb/IIIa complex, the highest density regions of which were in fractions 15–18 in this experiment. Human IgG was concentrated in fractions 12–14. In contrast, soybean trypsin inhibitor (mol wt 21,500) and transferrin (mol wt 76,000) remained in the upper part of the gradient (fractions 1–12). These results suggest that the bulk, at least, of the IIb/IIIa complex is of a limited size with a sedimentation coefficient lower than that of fibrinogen.

DISCUSSION

Interest in membrane glycoproteins IIb and IIIa intensified when it was shown that these glycoproteins were missing or severely reduced in concentration in membranes of platelets isolated from patients with Glanzmann’s thrombasthenia. In this bleeding disorder, platelets do not support aggregation as normally induced by any of the physiologic agents, such as ADP, thrombin, or collagen. It seemed reasonable to assume that the absence of aggregation was connected with the membrane glycoprotein defect. This assumption received support when it was demonstrated that an IgG alloantibody (IgG . . L), isolated from a polytransfused patient with thrombasthenia, inhibited platelet aggregation and specifically interacted with GP IIb/IIIa during crossed immunoelectrophoresis. The nature of the functional defect in thrombasthenia suggested that IIb and/or IIIa may play a role in the mediation of the platelet–platelet adhesion that concludes the aggregation mechanism. The finding that thrombasthenic platelets were unable to bind fibrinogen in the presence of ADP led to the proposal that the missing glycoproteins play a role in the expression of the fibrinogen receptor. In support of this hypothesis, Nachman and Leung have shown that complex formation between purified GP IIb and IIIa in vitro is accompanied by their acquisition of a fibrinogen-binding capacity.

In view of their possible role in platelet aggregation, more information is required concerning these glycoproteins and the nature of their organization in the normal human platelet membrane. Recently, Leung et al. performed a computerized analysis of autoradiographs of two-dimensional tryptic $^{125}$I peptide maps obtained using purified GP IIb and IIIa. Results suggested that the two glycoproteins had different structures and were separate molecular entities. The results of our present study, performed using density
gradient ultracentrifugation over 10%-40% sucrose gradients, have now confirmed that under certain conditions, GP IIb and IIIa associate together. Furthermore, additional evidence has been obtained to show that complex formation is mediated by divalent cations. When ultracentrifugation was performed using samples solubilized from membranes that had been incubated with 5 mM EDTA, neither IIb nor IIIa penetrated far into the gradient. However, when centrifugation was performed without divalent cation chelation, a distinct and characteristic change in the sedimentation profile of GP IIb and IIIa was observed. In agreement with the hypothesis that they were part of a higher molecular weight complex, the glycoproteins penetrated well into the gradient during ultracentrifugation. All platelets used in the present study were washed in EDTA-containing buffers prior to membrane isolation or TX-100 solubilization. It appears that externally added EDTA is unable to dissociate the IIb/IIIa complex as we have located it. Dissociation of the complex may be achieved by adding EDTA to TX-100-soluble extracts. However, at 4°C, dissociation is slow. Alternatively, EDTA may be added during TX-100 solubilization, or as in the present study, isolated membranes may be incubated with divalent cation-chelating agents prior to the addition of TX-100. This may mean that the divalent cation-binding sites are located at the cytoplasmic surface of the membrane. Alternatively, small amounts of bound or residual EDTA could effect the dissociation after the addition of the TX-100.

The above discussion assumes that the complex is present in the native platelet membrane. It is important to raise another alternative—that the complex is formed during platelet isolation, membrane fractionation, or TX-100 solubilization. Changing the platelet washing procedure, such as by substituting PGE1 for EDTA, had no effect on the appearance of the IIb/IIIa immunoprecipitate as subsequently located by CIE (unpublished studies). However, given the dissociating effect of EDTA, we were unable to perform TX-100 solubilization or membrane isolation under conditions that prevented the membrane glycoproteins coming into contact with internal subcellular stores of divalent cations, and thus cannot exclude the possibility that complex formation may occur during these procedures. Comparison of Figs. 2 and 3 shows that the altered sedimentation of GP IIb and IIIa, depending on whether or not divalent cation chelation was performed, was a phenomenon characteristic for IIb and IIIa. A nonspecific association of membrane glycoproteins and proteins during TX-100 solubilization can be ruled out as an explanation for our findings. SDS-PAGE analysis of the gradient fractions for 125I-labeled glycoproteins did, however, reveal evidence of the presence of additional glycoprotein complexes following TX-100 solubilization. In particular, Fig. 3A suggests the presence of an EDTA-resistant complex involving GP Ia. The close association of such complexes with IIb/IIIa is thought unlikely, since no traces of other major 125I-labeled membrane glycoproteins were revealed after SDS-PAGE analysis of the band 16 immunoprecipitate eluted from agarose gels following CIE. Platelets contain a protease activity that is stimulated by Ca2+. It was important to exclude that GP IIb/IIIa association was a result of Ca2+-dependent protease activity when platelets were lysed in the absence of EDTA. Leupeptin is a potent inhibitor of this enzyme. Incorporation of leupeptin into the lysis buffer during platelet solubilization by TX-100 resulted in no alterations in the shape or form of the IIb/IIIa immunoprecipitate as observed after CIE. In our original studies we showed that Ca2+, but not Mg2+, caused an apparent reassociation of IIb and IIIa when the divalent cations were added in excess to TX-100-solubilized platelet proteins previously incubated with EDTA. Thus, it appears that Ca2+ rather than Mg2+ is involved in IIb/IIIa complex formation. However, we have not yet shown that either IIb or IIIa themselves directly bind Ca2+. The possibility that other membrane components, phospholipids or low molecular weight polypeptides contribute to and mediate the complex formation cannot be excluded. Studies on the purified complex will be required to adequately answer this point. It is interesting, however, that a recent report has shown that platelets isolated from two patients with thrombasthenia showed a markedly decreased capacity to accumulate calcium. This observation may, or may not, be related to our findings, but it does indicate that thrombasthenic platelets are deficient in at least one of the probable multiple binding sites for Ca2+ in the platelet membrane. Further studies to identify these sites are required.

The criteria for identifying CIE immunoprecipitates containing either free GP IIIa, free GP IIb, or the complex are given elsewhere and are based on the shape of the precipitate and the distance of migration in the first dimension. Interestingly, it has recently been shown that the alloantibody, IgG.LL, which precipitates the complex during CIE, was unable to precipitate GP IIb and IIIa individually. This finding suggests that the complex itself gives rise to unique antigenic sites and may imply a close association between the two glycoproteins. A preliminary attempt has been made to define the size of the complex.
SDS-PAGE revealed that the most densely staining IIb/IIIa bands in those gradient fractions containing the complex were located between those of IgG and fibrinogen when the protein standards were centrifuged in an identical manner. This showed that the bulk of the complex was of limited size. Examination of Fig. 2B reveals that the CBR-stained bands representing IIb and IIIa in gradient fractions 11-16 were of similar intensity. In contrast, after EDTA treatment, an asymmetry is to be noted in the distribution of IIb and IIIa within the gradient. GP IIIa was relatively enriched in fraction 5 and GP IIb in fraction 12 (Fig. 2A). This may be interpreted as meaning that in their dissociated states, GP IIb, as a glycoprotein of slightly higher molecular weight (see Table 1), has penetrated further into the gradient than has IIIa. The lack of any similar finding in the absence of Ca²⁺ chelation suggests that IIb is complexing with IIIa in a stoichiometric manner. Taken as a whole, our data would suggest the probable presence of a IIb–IIIa heterodimer as the major component of the complex. Further studies on isolated glycoproteins are now required to confirm these data. Molecular weight estimates following density gradient ultracentrifugation in the absence of Ca²⁺ chelation are known. Only be accurately interpreted as meaning that in their dissociated states, GP IIb, as a glycoprotein of slightly higher molecular weight.

In conclusion, we have presented evidence that human platelet membrane GP IIb and IIIa may associate one with another in a specific manner. Further studies are now required to define whether part or all of the complex is an intrinsic component of the circulating platelet membrane, or whether it is formed subsequent to the taking of the blood sample. In a recent report, Polley et al. studied the topographical relationship between GP IIb and IIIa in stimulated and unstimulated human platelets. Monospecific rabbit antibodies to the purified glycoproteins were labeled with ferritin or keyhole limpet hemocyanin. Using the double labels and immunoelectron microscopy, it was demonstrated that GP IIb and IIIa formed clusters after platelet stimulation with thrombin. It was also shown that these complexes did not include GP I. The report of Polley et al. suggests that the complex, as we have described it, may represent an endpoint of platelet activation. We are continuing our studies into the organization of IIb and IIIa in the platelet membrane and into the possible functional significance of IIb/IIIa complex formation.

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Further studies on the interaction between human platelet membrane glycoproteins IIb and IIIa in triton X-100

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