Isolation of a Subpopulation of Glycoprotein IIb-III from Platelet Membranes That Is Bound to Membrane Actin

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ABSTRACT Triton X-100-insoluble residues, or skeletons, of plasma membrane-rich vesicles obtained from unstimulated human platelets were isolated by high speed centrifugation. About 10–15% of the total surface iodinatable glycoproteins IIb and III (GPIIb and GPIII, respectively) co-isolated with the insoluble fraction. After sonication and centrifugation the solubilized material was further purified by affinity chromatography on Lens culinaris lectin-Sepharose. SDS PAGE analysis of this material revealed the presence of at least three major proteins, which were shown to be GPIIb, GPIIa, and membrane actin, as judged by their electrophoretic properties and on the basis of immunological criteria. Antibodies directed against platelet surface glycoproteins and antibodies directed against rabbit actin were able to immunoprecipitate all three proteins, which indicates that they were noncovalently associated with one another. Gel filtration of the Lens lectin-purified Triton-insoluble complex on Ultrogel AcA 22 showed that >85% of the total surface GPIIb and III was associated with an actin-rich peak that eluted in the void volume. In contrast, the form of GPIIb-III present in the Triton-soluble membrane fraction behaved as monomeric species when chromatographed under identical conditions. Finally, the GPIIb-III membrane actin complex bound with high efficiency to rabbit f-actin in vitro in a Ca**+-independent manner, whereas the monomeric forms found in the Triton-soluble fraction did not bind to actin. These results indicate that two forms of GPIIb and III exist: one that binds directly to endogenous membrane actin and one that does not.

Numerous studies have provided largely indirect evidence for transmembrane interactions among surface proteins and receptors of cells and an internal membrane matrix composed of actin and other cytoskeletal proteins (3, 5, 7, 10, 14, 16, 17, 19, 23, 25, 27, 31, 35, 40, 45). Indeed, a number of studies have shown that the surface topography and lateral mobility of receptors can be modulated by the underlying cytoskeleton and its associated contractile and motile elements (20, 28, 30, 31, 36, 37, 39).

In the erythrocyte membrane, the major membrane glycoprotein, band III, interacts directly with ankyrin (2), which links this surface protein to the spectrin-actin submembranous matrix of this cell type (9, 27). The molecular basis for these interactions is poorly understood in nonerythroid cell types, however. One recent report has provided evidence that a major glycoprotein present in microvilli isolated from mammary adenocarcinoma cells is directly associated with actin (5, 18). However, the function of this glycoprotein is not yet known, so to relate these findings to receptor function is impossible at present. We have previously shown that a molecular complex exists in membranes derived from unstimulated platelets that consists of actin, glycoproteins IIb and III (GPIIb and GPIII, respectively),1 and polypeptides of 180,000–200,000 (29). We further demonstrated that these proteins can be crosslinked to one another in intact and detergent-extracted membranes by protein crosslinking agents (29). In addition, other work indicates that stimuli such as concanavalin A or thrombin can under the appropriate conditions induce a large increase in the total surface GPIIb and III (which bind concanavalin A [24]) recovered in the Triton-insoluble cytoskeleton (30, 35). These results indicated that even in the resting platelet membrane, a subfraction of GPIIb and III that accounted for ~10 to 15% of the total was already associated with the Triton-insoluble fraction of isolated membranes. These findings suggested the possibility of selective interaction of a subpopulation of GPIIb and III with the membrane skeleton and implied that a biochemical difference may exist that accounts for the different binding properties of the two glycoprotein populations.

1 Abbreviations used in this paper: GPIIb, GPIIa, and GPIII, glycoproteins IIb, Ib, and III, respectively; αMM, α-methyl-α-mannopyranoside.
In this paper, we report the isolation of a complex derived from the Triton-insoluble fraction composed of GPIIb, GPIII, and membrane actin. Unlike the GPIIb-III isolated from the Triton-soluble fraction, the complex binds with high affinity to f-actin. We believe that this represents the first reported Triton-soluble fraction, the complex binds with high affinity and membrane actin. Unlike the GPIIb-III isolated from the evidence that indicates that GPIIb-III is the receptor for fibrin membrane between a surface constituent and actin that can isolation of a molecular complex from a nonerythroid cell contractile apparatus. This physical attachment coupled with myosin light chain phosphorylation (11) and a subsequent centripetal actomyosin contraction (30) would provide for retraction of the fibrin clot.

MATERIALS AND METHODS

Reagents and Chemicals: Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt (41). Rabbit anti-chick actin was purchased from Miles-Yeda (Elkhart, Indiana). Protein A-Sepharose 4B was obtained from Sigma Chemical Co. (St. Louis, MO).

Cells: Human platelets were prepared from fresh human blood obtained by venipuncture from normal human volunteers. The platelets were isolated from platelet-rich plasma by centrifugation and further purified by gel filtration on Sephereose 2B (42). Platelets were surface iodinated with 125I by the lactoperoxidase method as previously described (31, 32). The labeled cells were washed by centrifugation and resuspended in Tyrode's balanced salt solution at a concentration of 4 x 10^9/ml.

Plasma Membrane-rich Fractions: Platelet plasma membrane fractions were prepared by the method of Barber and Jamieson (1). Monoclonal Antibody (PMI-1) against GPIIIa: The mouse monoclonal antibody (PMI-1) was prepared and thoroughly characterized elsewhere by Shadle et al. (38) and was kindly provided by Dr. Mark H. Ginsberg (Scripps Clinic and Research Foundation, La Jolla, CA). In brief, this monoclonal antibody was derived from mice immunized with platelet plasma membranes and was selected on the basis of its ability to inhibit platelet adhesion to collagen. Western blot analysis and immunoprecipitation studies established that the antigenic epitope resided on the glycoprotein IIb alpha (GPIIbalpha) subunit. Under conditions where GPIIbalpha was complexed to GPIII, the antibody also coprecipitated this component. This antibody is an IgG1 class antibody.

As a control an irrelevant mouse monoclonal of the same subclass was used with the ascites dilutions chosen so that total IgG concentration was identical. The ~32-labeled material was then applied to an Ultrogel AcA-22 (LKB Instruments, Inc., Gaithersburg, MD) column equilibrated with 10 mM sodium phosphate, 0.1 KCI, 1 mM EDTA, 0.04% Triton X-100 (pH 7.2). All samples were adjusted to the same final Triton X-100 concentration (2%) before application to the column.

Immunoprecipitation Assays: 125I-labeled material eluted from the lentil lectin-Sepharose 4B column with α-MM was incubated with either rabbit anti-chick or mouse IgG1 antibody against GPIII (GPIII) or with a comparable concentration of an irrelevant monoclonal antibody as indicated in the text. 3 mg of Protein A-Sepharose 4B prewollen with buffer was then added and incubated for 1 h at room temperature and then allowed to settle for 15 min at unit gravity. The supernatant fraction was removed and the beads were washed twice with buffer. The washed beads were treated with SDS PAGE sample buffer and then analyzed by SDS PAGE gel electrophoresis as described below.

Actin-binding Assay: Samples prepared as described in the text were incubated at room temperature for 30 min with 244 mg/ml of rabbit skeletal muscle actin (final concentration) under conditions as described by Cooper and Pollard (8). The sedimentable actin and any associated proteins were isolated by layering of the reaction mixture on a cushion of 30% sucrose in 10 mM sodium phosphate, 0.1 M KCI, 1 mM EDTA, 0.1% Triton X-100 (pH 7.2) and centrifuged in a Beckman Airfuge (Beckman Instruments Inc.) at 100,000 x g for 15 min. After centrifugation, the supernatant and pellet fractions were analyzed by SDS PAGE gel electrophoresis as described below.

SDS-PAGE Analysis: One dimensional 8% or 6-15% linear gradient SDS PAGE gels with slab gels were performed by the method of Laemmli (21) in a minigel system (41).

Samples of large volume were precipitated by the addition of an equal volume of 20% trichloroacetic acid for 30 min at room temperature. Samples were then centrifuged and washed twice with an equal volume of acetone. SDS PAGE sample buffer was then added and samples were analyzed as described above by SDS PAGE. Slab gels were stained for protein with Coomassie Brilliant Blue G-250, dried on a Bio-Rad Slab Gel dryer (Bio-Rad Laboratories), and autoradiographed as previously described (31). Relative protein contents of the gels were determined by densitometry of the wet Coomassie Blue-stained gels or the autoradiograms as indicated in the text as previously described (31).

Electroblot Analysis: Electrophoretic transfer of protein bands from SDS PAGE gels to nitrocellulose and subsequent antibody staining were performed as outlined by Towbin et al. (44). Binding of the primary test antibody was visualized after incubation with peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories) as described by Hawkes et al. (13).

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FIGURE 1 Extraction of membranes obtained from unstimulated human platelets with Triton, EDTA, Mg, ATP buffer. Membranes (PM) were treated with Triton-EDTA-Mg-ATP buffer and centrifuged as described in the Materials and Methods. The resultant pellet (TxP) contained ~10-15% of the total surface iodinated GPIII and III, and the Triton supernatant (TxS) contained the remainder. The Triton-insoluble pellet was subjected to sonication and recentrifuged. The supernatant (Son TxP) S contained >85% of the material originally present in the Triton-insoluble pellet. This figure shows 8% acrylamide SDS PAGE stained with Coomassie Blue.
RESULTS AND DISCUSSION

Isolation of GPIIb and III from the Triton-insoluble and Triton-soluble Fractions of Platelet Membranes by Chromatography on Lens Lectin-Sepharose 4B

Platelet membranes obtained from 10 U of freshly prepared platelet-rich plasma were extracted with 1% Triton X-100 in the presence of 5 mM EDTA and 2 mM Mg²⁺-ATP. The latter was included to remove myosin from the actin-rich insoluble fraction. The resulting extract was subjected to centrifugation (45,000 g, 30 min at 4°C) and the Triton pellet and supernatants were harvested. The platelets were surface iodinated with ¹²⁵I by the lactoperoxidase method (33) before preparation of the membranes. As shown in Fig. 1, the Triton pellet fraction is composed of actin, GPIIb and III, and numerous other components, most notably a doublet of proteins with Mr's of 190,000 and 220,000. The supernatant fraction on the other hand contains ~85% of the total membrane GPIIb and III and 10% of the total actin, as well as other proteins present in trace amounts. The identification of GPIIb and III was based on the relative mobilities of these peptides in SDS under both reduced and nonreduced conditions (6, 33), their lectin binding properties (24), and immunological criteria (see below).

The Triton pellet fraction was resuspended in Triton-EDTA buffer and subjected to sonication. After this treatment, >85% of the total GPIIb and III initially present in this fraction did not sediment when the sonicate was recentrifuged as before. This sonicated fraction together with the Triton soluble fraction was further purified by affinity chromatography on Lens lectin-Sepharose 4B. In the case of the sonicated Triton pellet fraction, a fraction that could be subsequently eluted with α-MM bound to the column. The material eluted with the lectin-specific saccharide was composed of GPIIb and III, as expected, and also a major polypeptide of 43,000 mol wt, which comigrated with actin on SDS PAGE under reduced conditions (Fig. 2A). Densitometric analysis of the Coomassie Blue-stained gels indicated that the three proteins were present in a molar ratio of 1:1:2.8, respectively, if it is assumed that the three proteins stain equally well with Coomassie Blue per unit mass.

In contrast, when the Triton-soluble supernatant fraction was subjected to Lens lectin chromatography, GPIIb and III and a trace of a surface-iodinatable protein of 135,000 mol wt were present with only minor traces of actin apparent (Fig. 2B).

Identification of the 43,000-mol-wt Polypeptide as Membrane Actin

Immunoprecipitation studies were performed to ascertain if the 43,000-mol-wt polypeptide, which co-isolated with
GPIIb and III, was actin. These studies were also designed to determine if the specific isolation of each constituent would result in the co-isolation of the other immunologically distinct proteins.

Fig. 3A shows that rabbit antibodies that crossreact with human actin, directed against chick actin, specifically immunoprecipitated actin and GPIIb-III when incubated with the affinity-purified Triton pellet fraction obtained as described in Fig. 2A. In addition, a monoclonal antibody (PMI-1) directed against GPIIb (38) also caused the co-immunoprecipitation of these three components. Furthermore, when 125I-labeled rabbit actin was added to the Triton-soluble forms of GPIIb-III, the respective antibodies immunoprecipitated only their respective antigens (data not shown).

Fig. 3B shows SDS PAGE analysis of PMI-immunoprecipitates on a 6–15% gradient gel. Similar results were obtained (see Fig. 3) when Triton X-100 (lane 1), deoxycholate (lane 2), or 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS; lane 3) was added to the immunoprecipitation reaction. Three major bands migrating with GPIIbα, GPIII, and actin were seen on this gel system. Furthermore, only a trace of a 20,000-mol-wt protein was noted that cocolcated with the β-subunit of GPII. Western blot analysis of the α-MM purified complex indicated that only the 43,000 band crossreacted with anti-actin (data not shown). These results indicate that the 43,000 peptide is immunologically related to actin and show further that the two surface glycoproteins are physically associated with the 43,000-mol-wt protein. The latter conclusion is supported by the co-isolation of actin with GPIIb-III by affinity chromatography (Fig. 2A) and by our previous finding that these proteins can be cross-linked to one another when isolated platelet plasma membranes are incubated with protein crosslinking reagents (29).

Characterization of the Complex between Actin and GPIIb-III by Gel Filtration on Ultrogel Ac22

The affinity purified fraction isolated from the sonicated Triton pellet as described in Fig. 2 A above was further characterized by chromatography on Ultrogel Ac22. For comparison the affinity-purified GPIIb and III isolated from the Triton supernatant fraction as described in Fig. 2B above were similarly analyzed. As shown in Fig. 4, the affinity-purified Triton pellet fraction eluted as a large peak of 125I surface label in the column void volume. SDS PAGE analysis indicated that >90% of the GPIIb-III initially present in the affinity purified material eluted in the void volume of the Ac22 column. In contrast, when the Triton soluble supernatant was analyzed in this way (Fig. 4B), >80% of the total GPIIb-III present eluted with a Stokes radius of about 61 Å, a value consistent with those reported by Jennings and Phillips for the monomeric forms of GPIIb and III (15). The remaining 20% eluted in the void volume with actin and presumably represents incomplete sedimentation of the complexes seen in the Triton pellet fraction.

To rule out the possibility that this fraction represented undissolved membrane bilayer fragments, we compared the buoyant densities of the void volume fraction with those of the monomeric fraction. Both forms had apparent buoyant densities in CsCl gradients of 1.38–1.40, so they did not differ significantly. In these gradients, intact plasma membranes banded at an apparent density of 1.26.

These results indicate that the complex between actin and GPIIb-III found in the Triton pellet fraction is large and probably asymmetric, and they support the idea that the GPIIb-III is associated with actin in the form of an asymmetric complex. More complete analysis is under way to determine the polymerization state of the actin present in the complex and the manner in which the GPIIb-III molecules are bound to the actin. In this regard it is interesting that the actin concentration is well below its critical polymerization concentration. Its presence in the void volume of the Ac22 column implies either that it is bound in the form of monomers to
Binding of the Isolated Complex to Rabbit f-Actin

The above results suggested that the Triton-insoluble form of the GPs was able to bind to actin and thus, potentially, to the platelet cytoskeleton. In contrast, the fraction of GPIIb-III found in the Triton-soluble fraction appeared to be monomeric even though actin was present in this fraction. In view of the large excess of actin in the membrane extract these results suggested that the supernatant GPs are unable to bind to actin. To test this directly, the purified complex and the monomeric form of GPIIb-III were incubated with filamentous rabbit actin and the actin was isolated by centrifugation in the airfuge. As shown in Fig. 5, in the presence of f-actin >90% of the complex form binds to the actin pellet. SDS PAGE analysis indicated that membrane actin, GPIIb-III, and traces (as judged by its Coomassie Blue staining intensity) of a 55,000-mol-wt protein bound to the rabbit actin. The complex alone did not sediment under these conditions. Furthermore, GPIIb and III in their monomeric form did not show any appreciable binding to rabbit actin even though the concentration of GPs used was higher than that present in the complex. The addition of enough Ca\(^{2+}\) to yield a free Ca\(^{2+}\) concentration of 2 mM did not affect the results obtained with either fraction (data not shown). These results show that the GPIIb-III present in the Triton supernatant is unable to bind to f-actin under the conditions employed and that the binding of the GPIIb-III-membrane actin complex binding to rabbit actin is mediated by the endogenously bound actin.

Since Ca\(^{2+}\) is required for heterodimerization of GPIIb and III in vitro (12, 15, 21, 34), our results suggest that the interaction of GPIIb-III with actin is controlled by another mechanism. Elsewhere we have shown that concanavalin A can induce the attachment of GPIIb and III to the platelet cytoskeleton by a passive process that is independent of cell energy (29), but that requires higher order clustering of GPIIb-III (31). These results support the possibility that transmembrane glycoproteins have low affinity sites for actin which when clustered, bind with very high affinity to actin, owing to the increased multiplicity of actin binding sites (4). Alternatively, GPIIb-III could interact with actin indirectly. This could be mediated by tightly bound lipids or a protein of small molecular weight. Analysis of PMI-1 immunoprecipitates on 6-20% gradient gels (Fig. 3 B) showed only trace amounts of a 20,000-mol-wt protein, which is absent under nonreducing conditions. This component is very likely the \(\beta\)-subunit of GPIIb, which is disulfide bonded to the larger \(\alpha\)-subunit (6). No other iodinatable protein bands were seen below a molecular weight of 10,000. Given our ability to chemically crosslink actin to GPIIb-III with 9-10 \AA-long .
bifunctional protein crosslinkers, we favor a model that does not involve a linkage protein. The possible involvement of tightly bound ligands cannot be excluded at this time and is currently under investigation.

In summary, the experiments presented in this paper indicate that GPIIb-III can exist in at least two different biochemical states. One of these represents in unstimulated membranes ~ 10–15% of the total and binds avidly to endogenous and exogenous actin filaments directly without the apparent intervention of a distinct linkage or anchoring protein like ankyrin or spectrin. The other form of GPIIb and III exists in monomeric form (in the presence of EDTA) with some tightly bound lipids cannot be excluded at this time and is associated with band 3 in human erythrocyte membranes. The fate of N-formyl chemotactic peptide receptors in human granulocytes: formation of slowly dissociating ligand-receptor complexes in transient association with the cell cytoskeleton. J. Cell Biol. 98:1378–1387.


