Characterization of a Platelet Membrane Protein of Low Molecular Weight Associated With Platelet Activation Following Binding by Monoclonal Antibody AG-1

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With the exception of the major platelet glycoproteins Iib/IIa and Ib, which function as receptors for fibrinogen and von Willebrand factor, little is presently known regarding the possible role of other platelet surface proteins in mediating platelet aggregation. We report the production of a murine monoclonal antibody (AG-i) recognizing human platelet membrane surface protein of relatively low molecular weight (mol wt) that may be involved in this process. AG-1 added to human platelet-rich plasma induces dense granule secretion and aggregation, with lag phase and maximal extent of aggregation dependent on antibody concentration. Aggregation induced by AG-1 is inhibited by AG-1 Fab fragments, indicating that the response is not Fc receptor-mediated. Although AG-1 continues to produce platelet shape change in the presence of EDTA, aggregation is fully inhibited and appears to be mediated by fibrinogen binding to glycoproteins Iib/IIa. AG-1 is a potent stimulus of thromboxane formation, but full inhibition of thromboxane production by 30 μmol/L indomethacin does not significantly inhibit platelet aggregation induced by 25 μg/mL AG-1, indicating that aggregation induced by AG-1 may proceed by way of an endoperoxide-independent pathway. Quantitation of AG-1 Fab binding to platelets reveals approximately 65,000 binding sites per platelet. When intact platelets are radioiodinated, immunoprecipitation of NP-40 lysates by AG-1 reveals an intensely labeled protein with an apparent mol wt of ~21,000 daltons, and several additional bands in the mol wt range of 22,000 to 28,000 daltons, all sharing the AG-1 epitope. These bands appear to be distinct from glycoprotein IX or from the β-chains of glycoprotein Ib or IIb. Finally, studies with platelets labeled by the periodate-β-hexosylamine procedure suggest the possibility of complex formation between subpopulations of glycoprotein Ib and the low-mol-wt glycoproteins recognized by AG-1.

The platelet membrane is the focus for molecular interactions involved in platelet activation and the platelet adhesion and aggregation responses. Platelet membrane glycoprotein Ib (GPIIb) serves as the receptor for von Willebrand factor and is responsible for platelet adhesion to vascular subendothelium. The membrane complex GPIIb/IIa serves as the fibrinogen receptor and is thought to mediate platelet aggregation. In contrast to these membrane glycoproteins, relatively little is presently known concerning the receptors for the various agonists that regulate platelet function and the signal-transducing elements of the membrane.

Studies using radiolabeled ligands have helped to establish that platelet membranes have specific receptors for a number of platelet stimuli, including thrombin, adenosine diphosphate (ADP), serotonin, epinephrine, and collagen. However, the membrane proteins that serve as the specific, functional receptors for these agents have not been conclusively identified. Bennett et al have identified a 100,000-dalton membrane-associated protein that is covalently labeled by the ADP affinity analog 5'-p-fluorosulfonylbenzoyl adenosine (FSBA). Incubation of platelets with FSBA resulted in the loss of platelet responsiveness to ADP, suggesting that the 100,000-dalton polypeptide was the ADP receptor involved in platelet activation. Studies with α-thrombin have suggested that the platelet membrane may have two distinct receptors. GPV has been shown to be a thrombin substrate that is preferentially cleaved from the platelet membrane. However, McGowan et al have found no consistent correlation between the degree of GPV hydrolysis and the extent of platelet activation. GPIib binds thrombin, and its absence in patients with Bernard-Soulier syndrome and modulation of its expression either by proteolysis or with antibodies correlate with decreased platelet responsiveness to α-thrombin. However, for both ADP and α-thrombin, a direct causal relationship between ligand binding to a specific membrane protein and platelet activation has not been demonstrated.

The present study focuses on membrane glycoproteins of relatively low molecular weight (mol wt) that may play a role in the processes leading to platelet activation. A new monoclonal antibody, AG-1, that recognizes such surface protein(s) is itself able to induce activation of platelets is described, and the membrane protein(s) characterized.

MATERIALS AND METHODS

Platelet function studies. For studies of platelet aggregation and adenosine triphosphate (ATP) secretion on citrated platelet-rich plasma (PRP), blood was drawn from normal volunteers and lumi-aggregation studies performed as previously described. Studies on washed platelets were performed on platelets prepared by the albumin density gradient method of Walsh et al. For studies of 5-hydroxytryptamine secretion, PRP prepared from blood collected in acid-citrate-dextrose was incubated for 30 minutes at 37 °C with 2 μmol/L [14C]5-hydroxytryptamine creatinine sulfate (Amersham, Arlington Heights, Ill) before washing and final resuspension in calcium- and magnesium-free Hanks solution (CMF-Hanks), pH 7.3, to which was added 1 mmol/L calcium (except in experiments where EDTA was used). Radioimmunoassay for thromboxane B2 generation by platelets in citrated PRP under nonstirred conditions.
was performed by the method of Granstrom and Kendahl as modified by Walenga et al.

Production of AG-1 monoclonal antibody and Fab fragments. BALB/c mice were immunized with washed platelets from patients previously diagnosed as having platelet-type von Willebrand's disease. Spleen cell suspensions prepared from the immunized mice were fused with P3 NS1.Ag 4.1 myeloma cells. Two weeks after fusion, culture supernatants were screened for their capacity to inhibit asialo von Willebrand factor-induced platelet agglutination of patient platelets in the presence of 5 mmol/L EDTA. Several culture supernatants were able to augment this response and on further testing were found capable of inducing aggregation of normal platelets without the addition of other agonists. One of these hybridomas (AG-1) was cloned by limiting dilution. The antibody was produced in ascites fluid of pristane-primed BALB/c mice and partially purified by precipitation with 50% saturated ammonium sulfate. After dialysis in phosphate-buffered saline (PBS), it was further purified by affinity chromatography on Affi-Gel Protein A (MAPS, Bio-Rad, Richmond, Calif). The immunoglobulin class of AG-1 was determined by Ouchterlony analysis using antisera purchased from Serotec (Bicester Oxon, England) and found to be an IgG of the γ1 subclass. Murine monoclonal antibodies AP-1 and AP-2, recognizing human platelet glycoproteins Ib and IIb/IIIa, respectively, were a kind gift of Dr Thomas Kunicki of the Southeastern Wisconsin Blood Center, Milwaukee.

For the preparation of Fab fragments, AG-1 monoclonal antibody at a concentration of 2.5 mg/ml was first digested with 25 μg/ml pepsin. After one hour at 37 °C, the digestion was stopped by adding 1/10 vol of 3 mol/L Tris, pH 9.0. The digest was reduced with 10 mmol/L cysteine and then alkylated with 25 mmol/L iodoacetamide before fractionation on a Sephadex G-150 superfine column (1.5 × 80 cm). The final AG-1 Fab pool was free of undigested IgG by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Indirect immunofluorescence. Peripheral blood mononuclear cells isolated by Ficoll-Hypaque density centrifugation or washed platelet lysates at concentrations of 10^7/ml were incubated with the primary monoclonal antibody or normal mouse serum at immunoglobulin concentrations of approximately 10 μg/ml for 30 minutes at 0 °C. After incubation with fluorescein-conjugated F(ab')2, fragments of goat antiserum IgG (Cappel, Malvern, Pa) and multiple washes, the cells were analyzed on a fluorescence-activated cell sorter (Coulter Electronic, Hialeah, Fla).

Scanning electron microscopy. Platelets in citrated PRP were prepared for scanning electron microscopy by fixation in 2.5% glutaraldehyde in White’s buffered saline for 20 minutes at room temperature. The fixed platelets were then centrifuged onto glass cover slips, rinsed in 0.9% NaCl, and dehydrated in graded ethanol solutions. Samples were transferred to acetone for five minutes and then critical point dried in carbon dioxide. The dehydrated samples were coated with gold and palladium using a Technics Hummer II (Technics, Alexandria, Va). Specimens were examined with a Hitachi scanning electron microscope at 25 kV.

AG-1 Fab binding. AG-1 Fab fragments were iodinated by the method of Fraker and Speck using Iodogen (Pierce Chemical Co, Rockford, Ill). Iodinated Fab was separated from free I131 using a Sephadex G-25 column equilibrated with PBS. The specific activity of the Fab was 1,550 cpm/μg protein as determined by absorbance at 280 nm.

After incubation of I131-AG-1 Fab with platelets in PRP for 30 minutes at room temperature, bound I131-AG-1 Fab was separated from free by centrifugation of the platelets through 20% sucrose in CMF-Hanks containing 2% bovine serum albumin. Nonspecific binding was assayed by measuring binding in the presence of 800 μg/mL undigested AG-1 antibody. The number of I125-AG-1 binding sites per platelet was obtained by Scatchard analysis of the binding data.

Radioiodination of platelets and immunoprecipitation of platelet lysates. To citrated PRP obtained from normal volunteers was added EDTA (13 mmol/L, final concentration), and the platelets were then washed three times in medium containing 154 mmol/L NaCl and 1 mmol/L EDTA, buffered to pH 7.4 with 10 mmol/L Tris. Platelets (10^9) were iodinated by the lactoperoxidase procedure with 1 mCi of NaI as described by Phillips and Aigin labeled by the periodate-[H]borohydride procedure.

For immunoprecipitation studies, labeled platelets at a concentration of 0.5 to 1.0 x 10^9/ml were solubilized with 0.5% (vol/vol) Nonidet P40 (NP-40) in the presence of iodoacetamide (10 mmol/L), phenylmethylsulfonyl fluoride (1 mmol/L), aprotinin (1%), and soybean trypsin inhibitor (25 μg/ml) (all obtained from Sigma Chemical Co, St Louis) to minimize proteolysis. The detergent lysates were centrifuged at 100,000 g for 60 minutes before immunoprecipitation. Lysates were incubated with 30 μg of the monoclonal antibody for 18 hours at 4 °C. The antibody and immune complexes were precipitated with goat antiserum immunoglobulin conjugated to agarose beads (Sigma). The beads were washed exhaustively in Tris-buffered saline with 0.1% NP-40 and the bound material eluted in 5% SDS buffered to pH 6.8 with 10 mmol/L Tris.

Electrophoretic analysis. One- and two-dimensional nonreduced-reduced SDS-PAGE of radiolabeled whole platelet lysates and immunoprecipitates was carried out using the discontinuous buffer method of Laemmli. I125-Labeled polypeptides were visualized by autoradiography with Kodak XRP-1 film and a Dupont Cronex intensifying screen (Dupont, Wilmington, Del.). I125-Labeled polypeptides were detected by fluorography after impregnating the gel with Enhance (New England Nuclear, Boston). Molecular weights were established by running marker proteins in parallel, with subsequent detection by Coomassie brilliant blue staining.

Electroimmunoblotting of platelet membrane proteins separated by SDS-PAGE was performed as described by Towbin et al. Proteins were transferred in 25 mmol/L Tris, 192 mmol/L glycine, 20% (vol/vol) methanol, pH 8.3 onto a nitrocellulose sheet. After blocking of the nitrocellulose sheet in 30 mmol/L Tris, 150 mmol/L NaCl, 0.05% (vol/vol) Tween 20, pH 7.3 (Bio-Rad, Richmond, Calif), the sheet was incubated first in AG-1 and then in peroxidase-conjugated goat antiserum IgG (Hyclone Labs, Logan, Utah). Peroxidase activity was visualized with 0.015% H2O2 (wt/vol) and 0.5 mg/mL 4-chloro-1-napthol (Sigma) in Tris-buffered saline.

RESULTS
Cellular specificity. Monoclonal antibody AG-1 was prepared by immunizing BALB/c mice with washed platelets from patients having platelet-type von Willebrand's disease, a disorder of hemostasis in which the platelet membrane shows an abnormally enhanced binding of von Willebrand factor. Although originally prepared against patient platelets, AG-1 has been found to be reactive with the platelets of all persons tested to date (>75%)

Binding of AG-1 to washed platelets from human subjects was demonstrated by indirect immunofluorescence. The platelets from normal subjects displayed a log normal distribution of fluorescence. The degree of staining was comparable to that produced by the monoclonal antibody AP-2, which recognizes the glycoprotein complex IIb/IIIa. Examination of peripheral blood lymphocytes and monocytes from the same individuals failed to reveal significant labeling.
Bone marrow aspirates were also examined microscopically by the indirect immunoperoxidase technique; only megakaryocytes and platelets stained with AG-1.

Characterization of AG-1-induced aggregation, secretion, and thromboxane B_2 production. The addition of AG-1 antibody to PRP resulted in strong platelet aggregation, ATP secretion, and thromboxane formation. At lower concentrations of AG-1, both aggregation and dense granule secretion were preceded by a characteristic lag phase (Fig 1). Over the concentration range of approximately 1.6 to 25 µg/mL (or higher), the total extent of platelet aggregation varied only slightly, although the lag phase varied considerably. Aggregation induced by 20 µg/mL AG-1 was not accompanied by lactate dehydrogenase release, indicating a noncytolytic mechanism for the aggregation and secretion responses. AG-1-induced platelet aggregation in PRP was not blocked by 30 µmol/L indomethacin, although ATP secretion was partially inhibited and thromboxane production was inhibited totally by the indomethacin. Apyrase at 5 U/mL (based on 5'-ATPase activity), a concentration that effectively blocks irreversible aggregation induced by ADP, gave only slight inhibition of AG-1-induced aggregation.

The aggregation response of AG-1-treated platelets was inhibited by 5 mmol/L EDTA. Typically, the addition of AG-1 to a suspension of stirring platelets in the presence of EDTA resulted in a decrease in light transmittance after a significant lag phase. Examination of the platelets by scanning electron microscopy before and three minutes after the addition of AG-1 confirmed that the observed decrease in light transmittance was the result of platelet shape change, with prominent pseudopod development (data not shown).

Fig 1. Induction of platelet aggregation and ATP secretion by AG-1. Citrated PRP (2 x 10^6 platelets per milliliter) was stirred at 1,200 rpm, 37 °C in the presence of 4 mg/mL luciferin-luciferase. Aggregation was monitored as increased light transmittance and ATP secretion as increased luminescence. AG-1 antibody was added at the arrow to a final concentration of (A) 0.78 µg/mL, (B) 1.56 µg/mL, (C) 3.13 µg/mL, (D) 6.25 µg/mL, or (E) 25.0 µg/mL.

Fig 2. Effect of AG-1 Fab on platelet aggregation induced by intact AG-1. Citrated PRP (2.2 x 10^6 platelets per milliliter) was stirred at 37 °C for two minutes in the presence or absence of 10 µg/mL AG-1 Fab. At the arrow, intact AG-1 antibody was added to a final concentration of 12.5 µg/mL, and aggregation subsequently monitored.

Fab fragments prepared from AG-1 antibody failed to induce platelet aggregation. These monovalent antibody fragments did, however, inhibit the response of platelets to intact AG-1 antibody (Fig 2). At lower ratios of AG-1 Fab to whole antibody the aggregation response was delayed, while higher ratios resulted in complete inhibition of aggregation. In contrast, AG-1 Fab fragments did not inhibit platelet aggregation induced by ADP (8 µmol/L), epinephrine (8 µmol/L), collagen (8 µg/mL), γ-thrombin (120 nmol/L), or calcium ionophore A23187 (25 µmol/L).

The addition of AG-1 to the PRP of a patient with Glanzmann’s thrombasthenia produced shape change and a normal amount of ATP secretion, but only a slight degree of aggregation—a pattern of responses identical to that seen in the patient with collagen or thrombin, suggesting a normal initial interaction of agonist with the platelets, but subsequent reduction in aggregation mediated by the platelet’s fibrinogen receptors (which are known to be decreased in this disorder). Furthermore, preincubation of normal PRP with monoclonal antibody directed against the GPIIb/IIIa complex also blocked AG-1-induced aggregation (Fig 3). Albumin density gradient-washed platelets did aggregate in response to AG-1 in the absence of fibrinogen; however, this
Table 1. Effect of Platelet Function Inhibitors on [14C]5-Hydroxytryptamine Secretion Induced by AG-1 or Thrombin

<table>
<thead>
<tr>
<th>Condition</th>
<th>AG-1 (Percentage of Cell Content)</th>
<th>Thrombin (Percentage of Cell Content)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>80.5 ± 3.9(4)</td>
<td>78.2 ± 6.0(4)</td>
</tr>
<tr>
<td>Dibutyryl cAMP</td>
<td>0.5 (2)</td>
<td>3.5 (2)</td>
</tr>
<tr>
<td>EDTA</td>
<td>9.7 ± 5.6(4)</td>
<td>84.3 ± 5.2(4)</td>
</tr>
<tr>
<td>Anti-GP Ib/IIa</td>
<td>2.0 (2)</td>
<td>81.5 (2)</td>
</tr>
<tr>
<td>Anti-GP Ib</td>
<td>80.0 (2)</td>
<td>86.5 (2)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>16.6 ± 7.8(3)</td>
<td>85.0 ± 5.3(3)</td>
</tr>
<tr>
<td>Adenosine</td>
<td>60.0 (2)</td>
<td>83.0 (2)</td>
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After uptake of [14C] 5-hydroxytryptamine by platelets, aliquots of washed platelets (1 x 10⁸/mL) were incubated at 37 °C for ten minutes in the presence of buffer alone, 2 mmol/L dibutyryl cAMP, 5 mmol/L EDTA, 20 μg/mL AP-2 (anti-GP Ib/IIa), 20 μg/mL AP-1 (anti-GP Ib), 30 μmol/L indomethacin, or 1 mmol/L adenosine before the addition of AG-1 (20 μg/mL) or thrombin (1 U/mL). Incubation was continued under nonstirred conditions for another ten minutes before the release reaction was stopped by the addition of formaldehyde to a final concentration of 1.5% (wt/vol). Aliquots of platelet-free supernatant were counted in a scintillation counter. Results are expressed as means ± SEM, with the number of experiments in parentheses.

response was accelerated when fibrinogen was added (data not shown). Antibody directed against GP Ib, which blocks vWF-mediated platelet agglutination induced by ristocetin, had no effect on AG-1-induced platelet aggregation (Fig 3).

Platelet-dense granule secretion induced by AG-1 was further studied by examining the secretion of [14C] 5-hydroxytryptamine from platelets. The results in Table 1 show that secretion induced by AG-1 occurred under nonstirred conditions and was inhibited by 5 mmol/L EDTA, 30 μmol/L indomethacin, 20 μg/mL AP-2, and 2 mmol/L dibutyryl cAMP. This contrasts with the [14C] 5-hydroxytryptamine secretion response induced by 1 U/mL thrombin, which was inhibited only by dibutyryl cAMP.

Binding of AG-1 Fab fragment to platelets. In order to estimate the number of sites recognized by AG-1 on platelets, binding studies were performed using 125I-iodinated AG-1 Fab that had been prepared by pepsin digestion. Figure 4 shows a representative saturation binding curve and the inset, a Scatchard analysis of the binding data from which the average number of binding sites per platelet was derived. Nonspecific binding assessed in the presence of excess unlabeled AG-1 never exceeded 8% of the total counts bound. Based on the analysis of seven normal human subjects, there were approximately 65,000 AG-1 Fab binding sites per platelet.

Electrophoretic characterization of the AG-1 antigen. NP-40 lysates of 125I-iodinated platelets were immunoprecipitated with monoclonal antibody AP-1 (directed against GPIb), AP-2 (directed against GPIb/IIa), or AG-1 and analyzed by 10% SDS-PAGE. On short exposures
Fig 6. Two-dimensional nonreduced-reduced SDS-PAGE of $^{125}$I-labeled platelets and AG-I immunoprecipitate. Whole lysate of $^{125}$I-iodinated platelets (A) and an AG-I immunoprecipitate of $^{125}$I-iodinated platelets (B) were electrophoresed nonreduced in a 12.5% polyacrylamide gel (left to right). After reduction with 2% β-mercaptoethanol, the polypeptides were separated in the second dimension on a 12.5% polyacrylamide slab gel (top to bottom). Labeled polypeptides were identified by autoradiography.

of the gels to x-ray film, the AG-I immunoprecipitates run under either nonreduced or reduced conditions revealed only a single band of approximately 21,000 daltons. Longer gel exposures revealed a series of at least four additional bands in the 22,000- to 28,000-dalton range (Fig 5A, lanes C and F). AG-I immunoprecipitates of the $^{125}$I-iodinated platelets did not reveal bands in the positions of GPIb (Fig 5A, lanes A and D) or of GPIIb/IIIa (Fig 5A, lanes B and E). On still longer exposure of the same gel, a 23,000-dalton polypeptide in the reduced GPIIb/IIIa immunoprecipitate became visible (Fig 5B, lane E). This band representing the β-chain of GPIIb (Jennings et al) is clearly distinct from the AG-I immunoprecipitate. Additional studies by two-dimensional nonreduced-reduced SDS-PAGE of $^{125}$I-labeled whole platelet lysates (Fig 6, panel A) and of AG-I-immunoprecipitated lysates (Fig 6, panel B) confirmed that the series of low-mol-wt polypeptides identified by AG-I are in fact the major iodinated platelet membrane glycoproteins in this mol wt range. In addition, the appearance below the diagonal of at least the 21,000-dalton polypeptide recognized by AG-I indicates a slightly increased mobility on reduction, suggesting the likelihood of intrachain disulfide bonds in this molecule.

The identity of the epitope recognized by AG-I was studied further by the Western blot technique. Lane A of Fig 7 is an autoradiograph of an $^{125}$I-labeled AG-I immunoprecipitate after SDS-PAGE and electrophoretic transfer to nitrocellulose. The major 21,000-dalton band, as well as the series of higher bands, was seen to transfer well to the nitrocellulose. When an unlabeled whole platelet lysate was similarly electrophoresed and transferred to the nitrocellulose, immunoperoxidase staining for epitopes recognized by
AG-I revealed an identical distribution (lane B). These bands were not detected if the platelet lysate was reduced before SDS-PAGE (lane C), or when blot studies of nonreduced platelet lysates were performed with isotypic control monoclonal antibodies substituted for AG-I. Two other bands located at approximately 35,000 daltons stained with AG-I under both nonreduced and reduced conditions but were also stained with a monoclonal antibody to GPIb (data not shown), indicating that these bands stained nonspecifically. Those studies thus indicated that each of the low-mol-wt bands identified in the AG-I immunoprecipitates of 125I-labeled platelets represents a polypeptide containing the epitope recognized by AG-I, rather than membrane components loosely attached to or coprecipitating with a single polypeptide of uniform mol wt.

The major 21,000-dalton band seen in AG-I immunoprecipitates of 125I-labeled platelets was seen only faintly when AG-I was used to immunoprecipitate platelets labeled by the periodate-[3H]borohydride technique (Fig 8, lane B), suggesting a relative absence of carbohydrate containing sialic acid. Bands in the 22,000 to 28,000 region were more prominent, reflecting a greater degree of glycosylation of these polypeptides. In both the nonreduced (lane B) and reduced (lane F) AG-I immunoprecipitates of 3H-labeled platelets, bands were present that comigrated with GPIb immunoprecipitated by monoclonal antibody AP-I (lanes A and E). The 17,000-dalton band (GPIX) coprecipitating with GPIb in AP-I immunoprecipitates (lane A) was also seen faintly in lane B, suggesting that GPIX (as well as GPIb) was in fact present in the AG-I immunoprecipitates. After reduction of the AP-I immunoprecipitate, the β-chain of GPIb could be identified at approximately 22,000 daltons and GPIX at approximately 20,000 daltons (lane E). AG-I immunoprecipitates of 125I-labeled platelets run alongside AP-I immunoprecipitates of 3H-labeled platelets under reducing conditions showed migration of these bands to be distinct from the major 21,000 band or the 22,000 to 28,000 bands recognized by AG-I (data not shown).

The results of the above experiments suggested the presence of GPIb in AG-I immunoprecipitates. However, precipitation of equal volumes of 3H-labeled platelet lysates with AG-I or with the anti-GPIb antibody AP-I resulted in 20 times more 3H in the AP-I immunoprecipitate. This indicates that only a small proportion (~5%) of the GPIb recognized by AP-I is found associated in AG-I immunoprecipitates.

In order to explore further the possible relationships between GPIb and the AG-I epitope suggested by the preceding studies, we performed immunoprecipitation of 3H-labeled platelets with AP-I or with AG-I, followed by precipitation of the residual supernatant with the other antibody. After initial immunoprecipitation of the platelet lysate with AG-I, subsequent immunoprecipitation of the "precleared" lysate with AP-I (Fig 8, lanes C and G) yielded bands indistinguishable from those resulting from AP-I immunoprecipitation alone. Of considerable interest was the repeated finding that when AP-I was used to "preclear" the platelet lysate, a subsequent AG-I immunoprecipitate (Fig 8, lane D) was virtually identical to the direct AG-I immunoprecipitate, except for absence of a single band—the high-mol-wt band migrating above the GPIb bands immunoprecipitated by AP-I. When the "precleared" immunoprecipitate was run under reducing conditions (Fig 8, lane H), the band pattern did not appear significantly different from that resulting from AG-I immunoprecipitation alone (Fig 8, lane F).

DISCUSSION

We have described platelet activation induced by a monoclonal antibody against human platelets and have characterized the membrane protein recognized by this antibody. Activation was initiated by a defined molecular interaction at the cell surface, the binding of antibody AG-I to its membrane epitopes. Activation was not mediated by the Fc region of the antibody, as indicated by the capacity of Fab fragment to block platelet aggregation induced by intact AG-I antibody. Aggregometry and scanning electron
stirred conditions. These results suggest that AG-i-induced activation was not the result of passive, antibody-mediated platelet agglutination. Incubation of platelets with AG-i showed that AG-i was capable of inducing platelet shape change in the presence of EDTA without appreciable agglutination. Microscopy showed that AG-1 was capable of inducing platelet shape change in the presence of EDTA without appreciable agglutination. Incubation of platelets with AG-1 resulted in a strong thromboxane response even under nonstirred conditions. These results suggest that AG-1-induced activation was not the result of passive, antibody-mediated platelet agglutination.

A strong dense granule secretion response was induced by AG-1 under nonstirred conditions. Whereas [3H]5-hydroxytryptamine secretion was only partially blocked by indomethacin, secretion was strongly inhibited by EDTA or by anti-GP-Ib/IIIa monoclonal antibody. In contrast, EDTA and anti-GP-Ib/IIIa antibody had no effect on thrombin-induced secretion. Recently, Powling and Hardisty26 have shown that an anti-IIb/IIIa monoclonal antibody (M148) could significantly inhibit Ca** influx and ATP secretion by platelets stimulated with ADP, platelet-activating factor, or low thrombin concentrations. They proposed the existence of a Ca** channel adjacent to the GP-Ib/IIIa complex that could be inhibited by anti-GP-Ib/IIIa antibodies. Another interpretation is that ATP secretion induced by weak agonists, including AG-1, is aggregation dependent and that even under nonstirred conditions, fibrinogen-dependent microaggregate formation occurs and potentiates secretion.

Microscopy showed that AG-1 was capable of inducing platelet shape change in the presence of EDTA without appreciable agglutination. Incubation of platelets with AG-1 resulted in a strong thromboxane response even under nonstirred conditions. These results suggest that AG-1-induced activation was not the result of passive, antibody-mediated platelet agglutination.
glycoprotein was not significantly affected by disulfide bond reduction. Although the implications of these findings remain unresolved, the highest-mol-wt band detected in the nonreduced AG-1 immunoprecipitates in the present study appears unlikely to represent the same structure. For, after reduction, this band disappears entirely, and only a single band with a mol wt identical to the GPIb alpha chain is seen in this region.

An intriguing finding in the present studies was that when 1H-labeled platelet lysates were first immunoprecipitated with AP-1, and these “precleared” lysates were subsequently immunoprecipitated with AG-1, the nonreduced AG-1 immunoprecipitates no longer revealed the highest-mol-wt bands. Removal of this band by AP-1 appeared selective, since AG-1 continued to precipitate 1H-labeled protein concomitantly with the GPIb bands seen in direct AP-1 immunoprecipitates. These results raise the interesting possibility that there may exist a previously unrecognized heterogeneity of GPIb molecules. The ability of AP-1 to remove the highest-mol-wt band suggests that this material does represent a form of GPIb. Those GPIb molecules persisting after pre-clearing by AP-1 may be complexed with the low-mol-wt peptides in such a way as to mask the AP-1 recognition site. Those GPIb molecules, however, that are associated with the highest-mol-wt band might possess a variant structure, such as variability in disulfide bonding pattern, that permits association with the low-mol-wt peptides recognized by AG-1, while still retaining exposure of the AP-1 recognition site.

Although our findings suggest that macromolecular associations may exist between GPIb and polypeptides recognized by AG-1, under the conditions of these studies only a relatively small percentage (approximately 5%) of the total pool of 1H-labeled GPIb recognized by AP-1 would actually appear to associate with the low-mol-wt polypeptides. Of this already small percentage, it is only those GPIb molecules that are actually precleared by AP-1 from the AG-1 immunoprecipitates that would be expected to be associated with the low-mol-wt polypeptides (ie, only 1% to 2% of the total GPIb recognized by AP-1). The absence of a significant 21,000-dalton band in AP-1 immunoprecipitates of 125I-labeled platelets, despite the relatively high labeling efficiency of this polypeptide for iodine, suggests that there may in fact not be a high degree of association of this polypeptide with GPIb. In contrast, GPIb may preferentially associate with the 22,000- to 28,000-dalton polypeptides that also bear the AG-1 epitope. Such a possibility would be consistent with the absence of low-mol-wt bands in AP-1 immunoprecipitates of 125I-labeled platelets, since these latter polypeptides label much less efficiently with iodine than does the major 21,000-dalton polypeptide. In view of the ability of monoclonal antibody AG-1 to induce platelet activation, further study of the molecular associations of GPIb with the series of low-mol-wt polypeptides recognized by AG-1 may well provide additional insights into platelet function.

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Characterization of a platelet membrane protein of low molecular weight associated with platelet activation following binding by monoclonal antibody AG-1

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