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

By Jonathan L. Miller, John M. Kupinski, and Kent O. Hustad

With the exception of the major platelet glycoproteins IIb/IIIa 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-1) 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. The response is not significantly inhibited by 25 μmol/L indomethacin does not significantly inhibit platelet aggregation induced by 25 μmol/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 radiiodinated, 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 distinc glycoprotein lb and the low-mol wt glycoproteins recognized by AG-1.

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 lumia- aggregation studies performed as previously described. Studies on platelets were performed on platelets prepared by the albumin density gradient method of Walsh et al. For studies on platelets prepared by the albumin density gradient method of Walsh et al. 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). Radioimmunossay for thromboxane B2 generation by platelets in citrated PRP under nonstirred conditions

THE PLATELET MEMBRANE is the focus for molecular interactions involved in platelet activation and the platelet adhesion and aggregation responses. Platelet membrane glycoprotein Ib (GPIb) serves as the receptor for von Willebrand factor and is responsible for platelet adhesion to vascular subendothelium. The membrane complex GPIb/IIIa serves as the fibrinogen receptor and is thought to mediate platelet aggregation. In contrast to these membrane glycoproteins, relatively little is 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. GPIb 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.

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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 \( \gamma 1 \) 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 \( \mu \)g/mL pepsin. After one hour at 37°C, the digestion was stopped by adding \( \frac{1}{10} \) 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 x 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 Ficol-Hypaque density centrifugation or washed platelets at concentrations of \( 10^7 \)/mL were incubated with the primary monoclonal antibody or normal mouse serum at immunoglobulin concentrations of approximately 10 \( \mu \)g/mL for 30 minutes at 0°C. After incubation with fluorescein-conjugated F(ab')2 fragments of goat antimouse IgG (Cappel, Malvern, Pa) and multiple washes, the cells were analyzed on a fluorescence-activated cell sorter (Coulter Electronics, 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. Critical point dried in carbon dioxide, the dehydrated samples were then critical point dried in carbon dioxide. The dehydrated samples were then critical point dried in carbon dioxide.

**AG-1 Fab binding.** AG-1 Fab fragments were iodinated by the method of Fraker and Speck using iodogen (Pierce Chemical Co, Rockford, Ill). \( 125I \)-Iodinated Fab was separated from free \( 125I \) on a 10-mL Sephadex G-25 column equilibrated with PBS. The specific activity of the Fab was 1,550 cpm/\( \mu \)g protein as determined by absorbance at 280 nm.

After incubation of \( 125I \)-AG-1 Fab with platelets in PRP for 30 minutes at room temperature, bound \( 125I \)-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 assessed by measuring binding in the presence of 800 \( \mu \)g/mL undigested AG-1 antibody. The number of \( 125I \)-AG-1 binding sites per platelet was obtained by Scatchard analysis of the binding data.

**Radiolabeling 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\(^7\)) were iodinated by the lactoperoxidase procedure with 1 \( \mu \)Ci of \( ^{125I} \), as described by Phillips and Agin or labeled by the periodate-[\( ^{131I} \)]borohydride procedure.

For immunoprecipitation studies, labeled platelets at a concentration of 0.5 to 1.0 \( \times 10^7 \)/mL were solubilized with 0.5% (vol/vol) Nonidet P40 (NP-40) in the presence of iodacetamide (10 mmol/L), phenylmethylsulfonyl fluoride (1 mmol/L), aprotinin (1%), and soybean trypsin inhibitor (25 \( \mu \)g/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 \( \mu \)g of the monoclonal antibody for 18 hours at 4°C. The antibody and immune complexes were precipitated with goat antimiouse 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. \( ^{125I} \)-Labeled polypeptides were visualized by autoradiography with Kodak XRP-1 film and a Dupont Cronex intensifying screen (Dupont, Wilmington, Del). \( ^{131I} \)-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 50 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 antimouse IgG (Hyclone Labs, Logan, Utah). Peroxidase activity was visualized with 0.015% \( H_2O_2 \) (wt/vol) and 0.5 mm/L 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). AG-1 has been found to be reactive with the platelets of all persons tested to date (>75% binding).
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 B2 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).

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 GPIb/IIa 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...
The number of Fab binding sites was determined. From which the number of Fab binding sites associated radioactivity was determined after centrifugation of washed platelets (1 x 10^8/mL) were incubated at 37 °C for 30 minutes without stirring. Platelet-mixed with increasing concentrations of labeled AG-i Fab and incubated at 22 °C for 30 minutes without stirring. Nonspecific binding was determined in the presence of buffer alone, 2 mmol/L dibutyryl cAMP, 5 mmol/L EDTA, 20 μg/mL AP-2 (anti-GPIIb/IIIa), 20 μg/mL AP-1 (anti-GPib), 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.

Platelet-dense granule secretion induced by AG-1 was further studied by examining the secretion of [14C]5-hydroxytryptamine, which was inhibited only by dibutyryl cAMP.

**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>Dibutryl 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-GPIb/IIla</td>
<td>2.0 (2)</td>
<td>81.5 (2)</td>
</tr>
<tr>
<td>Anti-GPib</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>
</tr>
</tbody>
</table>

After uptake of [14C]5-hydroxytryptamine by platelets, aliquots of washed platelets (1 x 10^8/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-GPIIb/IIIa), 20 μg/mL AP-1 (anti-GPib), 30 μmol/L indomethacin, 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.

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Platelet-dense granule secretion induced by AG-1 was further studied by examining the secretion of [14C]5-hydroxytryptamine, which was inhibited only by dibutyryl cAMP.
Fig 6. Two-dimensional nonreduced-reduced SDS-PAGE of $^{125}$I-labeled platelets and AG-1 immunoprecipitate. Whole lysate of $^{125}$I-iodinated platelets (A) and an AG-1 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-1 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-1 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)25 is clearly distinct from the AG-1 immunoprecipitate. Additional studies by two-dimensional nonreduced-reduced SDS-PAGE of $^{125}$I-labeled whole platelet lysates (Fig 6, panel A) and of AG-1–immunoprecipitated lysates (Fig 6, panel B) confirmed that the series of low-mol-wt polypeptides identified by AG-1 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-1 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-1 was studied further by the Western blot technique. Lane A of Fig 7 is an autoradiograph of an $^{125}$I-labeled AG-1 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-1 Western blots of platelet membrane proteins electrophoresed under nonreduced (lane B) and reduced (lane C) conditions.
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-1, 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 (data not shown), indicating that these bands stained nonspecifically. These bands were present that comigrated with GPIb in AP-I immunoprecipitates (lane A) was also faintly in lane B, suggesting that GPIb (as well as GPIIb) 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 GPIIX 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-1 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-1 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-1 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-1 (Fig 8, lanes C and G) yielded bands indistinguishable from those resulting from AP-1 immunoprecipitation alone. Of considerable interest was the repeated finding that when AP-1 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-1. 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-1 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-1 antibody. Aggregometry and scanning electron
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.

AG-1 Fab fragments inhibited platelet aggregation induced by low concentrations of whole AG-1 antibody and delayed the onset of aggregation induced by higher antibody concentrations, a finding that is consistent with a requirement for cross-linking of the membrane antigen recognized by AG-1. The failure of the AG-1 Fab fragment to fully inhibit aggregation induced by high AG-1 concentrations probably reflects dissociation of the Fab fragments by and increased membrane binding of the intact AG-1.

A strong dense granule secretion response was induced by AG-1 under nonstirred conditions. Whereas [³⁸⁰]S]-hydroxytryptamine secretion was only partially blocked by indomethacin, secretion was strongly inhibited by EDTA or by anti-GPIIb/IIIa monoclonal antibody. In contrast, EDTA and anti-GPIIb/IIIa antibody had no effect on thrombin-induced secretion. Recently, Prowling and Hardisty²⁹ have shown that an anti-IIb/IIIa monoclonal antibody (M148) could significantly inhibit Ca²⁺ influx and ATP secretion by platelets stimulated with ADP, platelet-activating factor, or low thrombin concentrations. They proposed the existence of a Ca²⁺ channel adjacent to the GPIIb/IIIa complex that could be inhibited by anti-GPIIb/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.

That AG-1 platelet activation may be under the control of intracellular cAMP is suggested by the inhibitory effect of dibutyl cAMP on AG-1-induced 5-hydroxytryptamine secretion. The potentiation of the aggregation response in the washed platelet system on the addition of fibrinogen, diminished aggregation response of thrombosthenic platelets to AG-1, and the inhibitory effect of an anti-GPIIb/IIIa monoclonal antibody (AP-2) on normal platelets suggest that AG-1-induced aggregation is in fact dependent on fibrinogen binding to GPIIb/IIIa.

Heterologous antihuman platelet antisera has been shown to cause in vitro platelet aggregation, ATP and 5-hydroxytryptamine release, and prostaglandin formation by a calcium-dependent mechanism that was not complement-mediated.²⁷-²⁹ The aggregation and secretion responses in these studies were not effectively inhibited by aspirin, suggesting an activation process different from that induced by ADP and epinephrine. These characteristics of the heterologous antiplatelet antibody-induced activation are similar to the AG-1-induced response described. However, the multispecificity of a heteroantisemur to whole cells makes this platelet activation response and its analysis complex.

Several groups have reported monoclonal antibodies against low-mol-wt membrane proteins of platelets that are capable of inducing platelet aggregation and secretion. Thiggarajan et al³⁰ briefly described an antibody to a 21,000-dalton polypeptide that could induce human platelet aggregation inhibitable by EDTA. Boucheix et al³¹ described a monoclonal antibody, ALB₆, prepared against human leukemic cells that could induce platelet aggregation, and Enouf et al³² showed that ALB₆ could induce calcium uptake by platelet membrane vesicles. The latter study showed that the 24,000-dalton protein recognized by ALB₆ was not identical to the low-mol-wt platelet protein involved in calcium transport that is phosphorylated by a cAMP-dependent protein kinase. During the course of the present study, Higashihara et al³³ reported on a monoclonal antibody to a 23,000-dalton platelet polypeptide that induced platelet aggregation and ATP secretion under stirring conditions. This antibody was also reported to block ristocetin-induced platelet aggregation. Jennings et al³⁴ have reported that several monoclonal antibodies against the β-chain of GPIIb induced platelet activation, and that Fab fragments of these antibodies were as effective as the whole antibody molecule.

We have shown that AG-1 recognizes platelet membrane polypeptides that are distinct from the β-chains of either GPIIb or GPIIb, as well from the 17,000-dalton (reduced) polypeptide associated noncovalently with GPIIb³⁵ that has been identified by some as GPIX.³⁶ The major AG-1-reactive polypeptide of approximately 21,000 daltons appears to be related to at least four larger, cross-reactive polypeptides that may represent glycosylation variants. These polypeptides appear to possess at least one intrachain disulfide bond, as suggested by the slightly increased mobility of the 21,000-dalton chain under the reducing conditions of two-dimensional nonreduced-reduced SDS-PAGE. The presence of such disulfide bonds is also suggested by the lability of the AG-1 epitope under reducing conditions, as demonstrated by Western blotting. Newman et al³⁶ have characterized a 24,000-dalton polypeptide found on a variety of acute lymphocytic leukemia cells and on some chronic lymphocytic leukemia cells. This protein (p24/BA-2) was glycosylated and had a pI near 7, which is similar to the polypeptide recognized by AG-1 (data not shown). However, the 24,000-dalton polypeptide described by Newman et al appeared to lack intrachain disulfide bonds. In addition, immunoprecipitation experiments failed to detect any molecular associations between this polypeptide and other membrane proteins of the leukemic cells.

In the peridate-[³⁵]H]borohydride-labeled AG-1 immunoprecipitates, bands were identified that comigrated with GPIb. The likelihood that this material does actually represent GPIb was further strengthened by the finding that the 17,000-dalton band (GPIX) coprecipitating with GPIb in the [³⁵]H-labeled AP-1 immunoprecipitates was identical in the AG-1 immunoprecipitates. In addition, a band migrating with a higher mol wt than those identified in the AP-1 immunoprecipitates was observed. Using wheat germ lectin affinity chromatography, Nachman et al³⁷ described a 210,000-dalton protein that these authors believed to be a structural analogue of GPIb. Kunicki et al³⁸ also demonstrated in the eluate from a wheat germ agglutinin column a fraction containing a high-mol-wt (190,000- to 210,000-dalton) glycoprotein. In both of these studies, the relative mobility in SDS-PAGE gels of the high-mol-wt...
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 3H-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 3H-labeled protein comigrating 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 preclearing 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 3H-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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