Interaction of AP-2, a Monoclonal Antibody Specific for the Human Platelet Glycoprotein IIb-IIIa Complex, with Intact Platelets*

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Dominique Pidard†, Robert R. Montgomery‡§, Joel S. Bennett¶||, and Thomas J. Kunicki†**
From the †Blood Center of Southeastern Wisconsin, Milwaukee, Wisconsin 53233 and the Departments of Microbiology and Pediatrics, Medical College of Wisconsin, Milwaukee, Wisconsin 53226 and the ‡Hematology-Oncology Section, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

A murine monoclonal antibody, designated AP-2, reacts specifically with the complex formed by human platelet membrane glycoproteins IIb and IIIa, but does not react at all with the individual glycoproteins. Purified AP-2 covalently coupled to Sepharose CL4B was used as an immunoadsorbent column to purify the IIb-IIIa complex from a preparation of Triton X-100-solubilized human platelet proteins. Radioiodinated AP-2 was shown to bind to a single class of sites, with 57,400 ± 9,700 molecules bound per cell (mean ± S.D.) at saturation and a dissociation constant ($K_d$) of 0.64 ± 0.15 nM (mean ± S.D.). Binding could not be readily reversed even after a 1-h incubation with a 100-fold excess of cold antibody. AP-2 inhibits ADP-induced binding of radiolabeled fibrinogen to gel-filtered platelets in a noncompetitive fashion, consistent with the previous observation that AP-2 also inhibits the aggregation of platelets in plasma induced by a number of physiologic agonists, including adenosine diphosphate, epinephrine, collagen, thrombin, and arachidonic acid.

Using AP-2, we have obtained evidence that the IIb-IIIa complex exists in the membrane of intact nonstimulated platelets and that complex integrity is not affected by external calcium ion concentration.

Human platelet glycoproteins IIb and IIIa form Ca$^{2+}$-dependent heterodimers in solution (1-3). Whether or not such heterodimers exist in the membrane of intact, nonstimulated platelets remains uncertain, although recent observations of Polley et al. (4), using immunoelectron microscopy, suggest that IIb-IIIa complex formation occurs only after platelet activation. A role for IIb and/or IIIa in platelet aggregation, particularly in the critical step of fibrinogen binding to activated platelets, has been postulated (5, 6) and recent evidence has demonstrated a direct and specific interaction between these glycoproteins and fibrinogen (7-9). To test the hypothesis that IIb and IIIa are physically separated in the membrane of nonstimulated platelets, and form heterodimers upon platelet activation, we have prepared a monoclonal antibody specific for the IIb-IIIa complex. Recently, we showed that this monoclonal antibody, AP-2, bound in a specific and saturable manner to platelets in anticoagulated whole blood (10). In the present report, we provide further characterization of the interaction between this antibody and its antigen(s) under a variety of conditions and the effects of this interaction upon selected platelet functions.

EXPERIMENTAL PROCEDURES AND RESULTS

DISCUSSION

By analyzing the association of radiolabeled antibody with preformed immunoprecipitates in cross-linked electrophoresis, we have been able to demonstrate that the murine monoclonal antibody, AP-2, recognizes an epitope located on the Ca$^{2+}$-dependent complex of glycoproteins IIb and IIIa and that it does not react with either free IIb or free IIIa (Fig. 1). Thus, the epitope recognized by AP-2 appears to be either a combinatorial determinant consisting of portions or each glycoprotein or a conformational determinant on one (or both) of the glycoproteins which is expressed only when it is coupled to the other. Using the same methodology, Woods et al. (19) previously identified other murine monoclonal antibodies specific for the complex of IIb-IIIa alone, or reactive with IIb whether free or complexed, and McEver et al. (20) recently characterized two murine monoclonal antibodies; Tah, which reacts with IIb (free or complexed), and T10, which reacts only with the IIb-IIIa complex. Using AP-2 immobilized on Sepharose CL4B, we purified the IIb-IIIa complex to homogeneity from Triton extracts of whole platelets by single step affinity chromatography (Fig. 2). No other proteins were copurified.

AP-2 specifically binds to a single class of sites on normal, unstimulated washed platelets, with a high affinity ($K_d < 1$ nM). At equilibrium, 57,000 AP-2 molecules were bound per platelet, in good agreement with values reported by others for monoclonal antibodies reactive against the same glycoprotein.
Platelet Membrane Glycoprotein IIb-IIIa

Under the conditions of our assay, the binding of $^{125}$I-AP-2 was, for all practical purposes, irreversible since an excess of unlabeled antibody could not displace bound labeled antibody even when the latter was incubated with platelets for only 30 s. This phenomenon did not require metabolically active platelets and was not a consequence of covalent cross-linking between murine IgG molecules and IIb and/or IIIa. The binding of fibrinogen to activated washed platelets has also been shown to become irreversible with time (6, 21, 22). Since the fibrinogen receptor is also closely associated to glycoprotein IIIa (8), it is conceivable that irreversibility of ligand-IIb-IIIa interactions is a reflection of unique properties of IIb-IIIa and independent of the nature of the ligand. One explanation for this phenomenon would be that conformational changes in IIb-IIIa which result from ligand binding dramatically decrease the dissociation (off rate) of such ligands. McEver and co-workers have obtained similar results with regard to the binding of T10 to human platelets. On the other hand, Diminno et al. (23) recently reported that the binding of another IIb-IIIa complex-specific murine monoclonal antibody was readily reversible and that even after a 120-min incubation with labeled antibody, greater than 90% of radioactivity could be displaced within 3 min by addition of excess cold antibody. For that antibody, one class of 21,000 binding sites was observed. These differences in binding kinetics most likely reflect differences in rates of association versus dissociation and are probably influenced to a large degree by epitope specificity.

We recently reported that AP-2 was a potent inhibitor of
platelet aggregation induced by ADP (5 μM), thrombin (0.1 unit/10^8 platelets), arachidonic acid (0.2 mg/10^8 platelets), epinephrine (20 μM), or collagen (1 μg/10^8 platelets) (24). Inhibition was dose-dependent with complete inhibition observed at ≥1.0 μg of AP-2 per 10^8 platelets. AP-2 did not inhibit either shape-change induced by any of the above reagents or ristocetin-induced agglutination of formalin-fixed platelets in the presence of plasma. On the other hand, AP-1, a monoclonal antibody specific for GPIb (10), had no effect on ADP and arachidonic acid-induced aggregation, but completely abolished ristocetin-induced agglutination. Neither AP-1 nor AP-2 directly agglutinated or aggregated platelets even at concentrations as high as 50 μg/10^8 platelets. Accumulated evidence demonstrates a crucial role for fibrinogen in platelet-platelet cohesion and aggregation (6, 8, 22) and indicates that IIb-IIIa is most likely the physiologic receptor for fibrinogen binding (7–9). It is certainly not coincidental that the amount of AP-2 required to completely abolish platelet aggregation (1.0 μg/10^8 platelets) is essentially identical with that amount shown in this report to produce maximal inhibition of fibrinogen binding (2.0 μg/10^8 platelets). These results further substantiate the hypothesis that platelet aggregation is mediated by the interaction of IIb-IIIa with fibrinogen.

Data presented in this report are consistent with the contention that glycoproteins IIb and IIIa exist as preformed complexes in the membrane of intact platelets subjected to a minimum of stimulation. We propose that platelet activation by ADP, thrombin, and probably other stimuli induces a conformational change in pre-existing complexes thus generating specific sites on IIb-IIIa for fibrinogen binding. Since complex integrity depends, to a certain extent, on the presence of calcium (1–3), but is independent of the concentration of external calcium when studies are performed with intact platelets, it is likely that calcium fluxes within the plasma membrane or in the cytoplasm mediate complex integrity and fibrinogen receptor induction.

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REFERENCES
Supplementary Material: Induction of AP-2, a Monoclonal Antibody Specific for the Human Platelet GPIb-IIIa Complex, with Intact Platelets

Diane L. Mason, Daniel J. O’Shea, Robert K. Bennett, and Thomas J. Kantik

Experimental Procedures

Triton X-100 and 

Affinity Chromatography

The preliminary characterization of AP-2 has been performed using 125I-labeled AP-2 in the presence of 125I-labeled AP-2 and in the absence of 125I-labeled AP-2. The results are presented in table 1.


determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and crossed immunoelectrophoresis (CIE) of selected fractions. SDS-PAGE and CIE analysis of eluted material showed that the affinity-purified material was characterized by single bands corresponding to polypeptides with apparent molecular weights (determined by SDS-PAGE) of 150,000 and 200,000. Each protein band was also observed by periodic acid-Schiff (PAS) staining and was identified by 

Quantitative Binding of 125I-AP-2 to Washed Platelets

Half-maximum binding of 125I-AP-2 to platelets was achieved in approximately 2 min., and binding was complete after 30 min. The 125I-AP-2 preparations used in these assays were homogenous, based on both SDS-PAGE analysis and the fact that more than 90% of the radioactivity was associated with the platelet pellet under conditions of 5.0% formalin fixation (12). Identical results were obtained with platelets from different donors, the number of AP-2 molecule bound per platelet was determined to be 1.3 x 10^9 (mean 220), with a dissociation constant (Kd) of 0.9 x 10^{-12} M (mean 6.0). In preliminary experiments (Figure 1), the amount of AP-2 bound was directly proportional to the concentration of antibody, reaching saturation at 125I-AP-2 (1.5 x 10^9 M) with an apparent dissociation constant of 0.64 x 10^{-12} M.

In a typical experiment (Figure 3), 3 x 10^9 washed platelets were incubated with increasing concentrations of 125I-AP-2, and the amount of radioactivity bound was determined by CIE analysis of eluted material. The data were fitted to a single-site binding model, and the number of AP-2 molecules bound per platelet was determined to be 1.3 x 10^9 (mean 220), with a dissociation constant (Kd) of 0.9 x 10^{-12} M (mean 6.0). In preliminary experiments (Figure 1), the amount of AP-2 bound was directly proportional to the concentration of antibody, reaching saturation at 125I-AP-2 (1.5 x 10^9 M) with an apparent dissociation constant of 0.64 x 10^{-12} M.

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TABLE 1
Platelet Membrane Glycoprotein IIb-IIIa

<table>
<thead>
<tr>
<th>Donor</th>
<th>Buffer alone</th>
<th>Buffer + 10 μg AP-2</th>
<th>Buffer + 5 μg AP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>n = 33,070</td>
<td>n = 35,350</td>
<td>n = 36,500</td>
</tr>
<tr>
<td></td>
<td>ED = 0.30</td>
<td>ED = 0.48</td>
<td>ED = 0.43</td>
</tr>
</tbody>
</table>

Addition of a 100-fold excess of unlabeled antibody either 30 sec., 2 min., or 10 min. after the initial addition of 125-I-AP-2 completely inhibited any further binding of 125-I-AP-2, but did not displace already bound 125-I-AP-2, even after a further 30 min. incubation (Figure 6). These experiments were then repeated using lyophilized, formalin-fixed human platelets reconstituted and washed in the same buffer and analyzed under identical conditions. The time course and extent of AP-2 binding were similar to that observed with non-fixed platelets, and no displacement of bound 125-I-AP-2 by unlabeled antibody was observed. Thus, binding is not dependent on metabolically active platelets. Platelet samples washed with buffer alone were reconstituted in buffer containing 5 mM EDTA, 50 μM PGE1, and 100 μM aspirin under reduced conditions. The percentage of 125-I-AP-2 associated with the pellet after 30 min. or 2 hr. under nonreduced conditions, the percentage of 125-I-AP-2 associated with the pellet after 30 min. under reduced conditions, and the percentage of 125-I-AP-2 associated with the pellet after 30 min. under reduced conditions were identical to that observed in blood anticoagulated with ACD alone. The use of any of a number of other anticoagulants, including heparin, citrate or EDTA-Mg-ATP (EMT), did not affect AP-2 binding.

Binding of 125-I-AP-2 to Platelets in Whole Blood

We previously reported that purified 125-I-AP-2 binds to normal and Bernard-Soulier platelets, but not to thrombocytopenic platelets, in citrated whole blood (10). Although these results suggested that the Ibb-IIIa complex is expressed on non-washed, non-citrate-fixed platelets in whole blood, we repeated such experiments with blood collected in acid-citrate-dextrose (ACD) anticoagulant containing 5 mM EDTA, 50 μM PGE1, and 100 μM aspirin in order to further minimize the possible influence of the citrate used. As shown in Figure 7, specific inhibition of fibrinogen binding (Figure 3) depended upon the results of these experiments using platelets from three different normal donors. AP-2 at 7 μg/ml inhibited AP-induced fibrinogen binding by 54% (mean). Inhibition was directly proportional to antibody concentration and was noncompetitive, the calculated Ki being 1 μg/ml.
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