Changes in the Platelet Membrane Glycoprotein IIb·IIIa Complex during Platelet Activation*

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Platelet activation is accompanied by the appearance on the platelet surface of approximately 45,000 receptor sites for fibrinogen. The binding of fibrinogen to these receptors is required for platelet aggregation. Although it is established that the fibrinogen receptor is localized to a heterodimeric complex of the membrane glycoproteins, IIb and IIIa, little is known about the changes in this complex during platelet activation that result in the expression of the receptor. In the present studies, we have developed and characterized a murine monoclonal anti-platelet antibody, designated PAC-1, that binds to activated platelets, but not to unstimulated platelets. PAC-1 is a pentameric IgM that binds to agonist-stimulated platelets with an apparent $K_a$ of 5 nM. Binding to platelets is dependent on extracellular Ca$^{2+} (K_{Ca} = 0.4 \, \mu M)$ but is not dependent on platelet secretion. Platelets stimulated with ADP or epinephrine bind 10,000-15,000 $^{125I}$-PAC-1 molecules/platelet while platelets stimulated with thrombin bind 20,000-25,000 molecules/platelet. Several lines of evidence indicate that PAC-1 is specific for the glycoprotein IIb·IIIa complex. First, PAC-1 binds specifically to the IIb·IIIa complex on Western blots. Second, PAC-1 does not bind to thrombasthenic platelets or to platelets preincubated with ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid at 37°C, both of which lack the intact IIb·IIIa complex. Third, PAC-1 competitively inhibits the binding of $^{125I}$-A2Aa, an IgG monoclonal antibody that is specific for the IIb·IIIa complex. Fourth, the antibody inhibits fibrinogen-mediated platelet aggregation. These data demonstrate that PAC-1 recognizes an epitope on the IIb·IIIa complex that is located near the platelet fibrinogen receptor. Platelet activation appears to cause a Ca$^{2+}$-dependent change involving the glycoprotein IIb·IIIa complex that exposes the fibrinogen receptor and, at the same time, the epitope for PAC-1.

Platelet activation is an ordered sequence of events that begins with the binding of an agonist to its receptor and ends with platelet shape change, aggregation, and secretion. Platelet aggregation requires the expression of receptors for fibrinogen on the platelet surface (1-5). While nonactivated platelets are unable to bind fibrinogen, activated platelets bind approximately 45,000 fibrinogen molecules/cell. Although the mechanisms that control the expression of fibrinogen receptors are unknown, the available evidence suggests that the receptors are formed entirely or in part by a heterodimer complex of the integral membrane glycoproteins, IIb and IIIa. 1) Thrombasthenic platelets, which lack glycoprotein IIb and IIIa, are unable to bind fibrinogen or aggregate (1, 3). 2) Fibrinogen coupled to photoactivatable cross-linking reagents selectively binds to IIb and IIIa (4, 5, 3). The solubilized IIb·IIIa complex binds to immobilized fibrinogen (6). 4) Monoclonal antibodies specific for the IIb·IIIa complex inhibit platelet aggregation (7-10). Physical conditions that dissociate IIb from IIIa within the membrane also inhibit fibrinogen binding and platelet aggregation (11, 12).

Glycoprotein IIb is composed of two disulfide-linked subunits of $M, 116,000$ and $23,000$. Glycoprotein IIIa is a $95,000$ polypeptide containing intramolecular disulfide bonds (13). The stability of the glycoprotein IIb·IIIa complex, both in detergent solution and within the platelet membrane, is dependent upon the presence of Ca$^{2+}$ (11, 12, 14-17). One potential explanation for the failure of fibrinogen to bind to resting platelets is that platelet activation results in the formation of the IIb·IIIa complex from its previously separate component parts. However, it appears that the IIb·IIIa complex is already present on the surface of unstimulated platelets. For example, monoclonal antibodies that recognize only the intact complex bind equally well to resting and stimulated platelets (7). Therefore, expression of the fibrinogen receptor during platelet activation must be due to formation of the complex per se, but to changes in the pre-existing complex. In the present studies, we have developed and characterized a new monoclonal antibody specific for the IIb·IIIa complex that binds preferentially to activated platelets. Our results suggest that changes in the conformation of the IIb·IIIa complex are necessary for expression of the platelet fibrinogen receptor.

**EXPERIMENTAL PROCEDURES**

Production and Screening of Monoclonal Antibodies—Platelets obtained from a patient with Glanzmann's thrombasthenia were used to immunize BALB/c mice. Splenic lymphocytes from these mice were fused to mouse myeloma SP-2 cells as described previously (8, 18). Hybridoma culture supernatants were screened for anti-platelet activity in a two-stage process. In the first stage, an enzyme-linked, immunosorbent assay (ELISA) was employed in which washed, normal platelets were applied to polyvinyl chloride microtiter wells using 0.001% poly-L-lysine and fixed with 0.25% glutaraldehyde. In preliminary studies, we determined that this process of washing and fixation of the platelets resulted in their becoming activated, at least to the extent that their fibrinogen receptors had become expressed. 

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Glycoproteins IIb-IIIa and Platelet Activation

The final substrate in the assay was orthophenylene diamine, and the reaction product was quantitated at 405 nm with a Multiscan spectrophotometer (18). Positive supernatants were retested in this ELISA using human lymphoid (HUT 78) and myeloid (K562) cells to exclude positive reactions that were not platelet-specific.

Cloned mouse hybridoma cultures that were positive in the first assay were examined in an ELISA designed to identify antibodies that reacted with activated platelets but not with unstimulated platelets. Whole blood was anticoagulated with 13 mM trisodium citrate, and platelet-rich plasma was obtained by differential centrifugation and then incubated for 20 min at 22°C with 1 mM aspirin to inhibit platelet cyclooxygenase. Platelets were separated from plasma by gel filtration on Sepharose 2B (Pharmacia Fine Chemicals) using an elution "gel filtration buffer" that contained 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 5.6 mM glucose, 1 mg/ml bovine serum albumin (Sigma), and 20 mM HEPES, pH 7.40 (19).

Two-hundred μl of the platelets (100 platelets/ml) were then placed into plastic microtiter wells and incubated for 5 min with either ADP (10 μM), epinephrine (10 μM), or thrombin (10 million units/ml) to stimulate the platelets or with 1 μM PGI2 to prevent platelet activation. After the 5 min, 50 μl of hybridoma culture supernatant was added to each well, and the mixtures were incubated for 20 min at 22°C. Platelets were cleaned by washing the membrane for 20 min with 5-10 ml of PBS, and applying the sample to a 1-chloro-4-naphthol solution. After the 5 min, 50 μl of hybridoma culture supernatant were added to each well, and the mixtures were incubated at 22°C for 30 min. Two hundred μl from each well were transferred to a 0.45-μm nitrocellulose membrane that had been presoaked in 6% bovine serum albumin. The membrane was incubated for 15 min with 1-chloro-4-naphthol (Sigma), according to Hawkes et al. (20), then washed with PBS, and air-dried. Anti-platelet antibodies yielded a blue reaction product that could be quantitated visually from 0 to 4+ or quantitated by reflectance densitometry.

Of the 380 culture supernatants obtained in the fusion, 25 showed platelet-specific reactivity using the first ELISA method. Where these 25 were tested further using the second ELISA method, one supernatant PAC-1, consisting of 3 to 5 antibodies from the plaque hybridoma cultures but only zero-trace with PGI2-treated platelets. This culture was cloned by limiting dilution and produced in quantity in mouse ascitic fluid.

**Purification and Characterization of Monoclonal Antibodies—**PAC-1 was shown to be an IgM using a mouse immunoglobulin subtype identification kit (Boehringer Mannheim). It was purified from 15 to 20 ml of ascites by precipitating the protein in 20 volumes of 2% nonidet p-40, incubating the mixture for 2h at 4°C, and then centrifuging the sedimenting detergent-insoluble residue, one hundred pg of solubilized protein were applied to a 5-20% polyacrylamide gradient slab gel and electrophoresed under conditions that separate the IIb-IIIa antigens (8).

**Identification of the PAC-1-specific Antigen on Platelets—**The PAC-1 antigen was identified on Western blots of nondenatured platelet proteins (12). Platelets were solubilized in a buffer containing 0.2% Triton X-100, 10 mM CHAPS, 0.1 mM leupeptin, 0.9 mM phenylmethylsulfonyl fluoride, and 5 mM Tris-HCl, pH 7.4. After sedimenting the detergent-insoluble residue, one hundred μg of solubilized protein were applied to a 5-20% polyacrylamide gradient gel electrophoresed under conditions that separate the IIb-IIIa complex from dissociated IIb and IIIa and from other platelet proteins (12). The platelet proteins were transferred from the gel to positively charged Zeta-blot paper (American Cyanimid) (23), and protein bands reacting specifically with PAC-1 were identified using peroxidase-conjugated goat anti-mouse immunoglobulin (20).

**Direct Binding of Monoclonal Antibodies to Platelets—**PAC-1 and A2A9 were radiolabeled with Na125I using Enzymobeads (Bio-Rad) and separated from free Na125I by gel filtration on Sephadex G-25 followed by overnight dialysis against PBS. The specific activity of the antibodies ranged from 150,000 to 250,000 cpm/μg protein. Labeled antibody co-migrated with the unabeled species on SDS gels. Furthermore, when varying proportions of PAC-1 and 125I-PAC-1 were added to ADP-stimulated platelets, the presence of a constant amount of 125I-PAC-1, as well as of 125I-PAC-1 plus unlabeled antibody, was the per cent of 125I-PAC-1 added was linear. This indicates that the unlabeled and labeled species interacted with platelets with similar affinities.

All steps in the binding assay were performed at room temperature. Unless stated otherwise, platelet-rich plasma was incubated with 1 mM aspirin for 20 min at 22°C, and the platelets were gel-filtered as described above. If necessary, the platelet concentration was adjusted to 0.5-1.0 x 10^11/ml, a range over which 125I-PAC-1 binding was linear with platelet count. The binding reaction was started by incubating platelets for 5 min with either PGI2 or with an agonist. Then duplicate or triplicate 500-μl aliquots were transferred to a final reaction tube containing 1-50 μl of 125I-labeled monoclonal antibody. The concentration of the PAC-1 or A2A9 used in each experiment was calculated from its absorbance at 280 nm (PAC-1: ε = 1.18 x 10^4 cm^-1 M^-1, A2A9: ε = 1.40 x 10^4 cm^-1 M^-1). The total amount of antibody bound to each reaction tube was determined by the radioactivity of the antibody preparation and the total counts/min of 125I in each reaction tube. After incubation of the platelets with the antibody for 5 min, platelets and bound antibody were separated from free antibody by filtration on 0.45-μm nitrocellulose filters that had been presoaked in 6% BSA. The reaction tubes were then rinsed with 4 ml of gel filtration buffer, and the filters were washed with three further 5-ml aliquots of buffer. All wash steps were complete within 15 s. Dried filters were counted for 125I in a γ-counter.

In preliminary studies, we determined that the absence of platelets, the filters retained less than 0.7% of applied 125I-PAC-1. The amount of platelet-bound antibody determined by this method was identical to that determined by a centrifugation method using silicone oil (19). The amount of free antibody in each reaction tube was determined by subtracting binding antibody from total antibody.

In some experiments, the effect of the antibody and of the agonist on the release of serotonin and platelet factor 4 from platelets was studied. Serotonin release was measured after platelets were loaded with [3H]serotonin, gel-filtered, and then incubated with unlabeled antibody and an agonist (19, 24). To measure platelet factor 4 release, gel-filtered platelets were incubated with the antibody and an agonist, then 1/3 volume of a platelet inhibitor solution (Thrombotect, Bectin-Dickinson, Rutherford, NJ) was added, and the tubes were incubated for an additional 30 min in an ice bath (25). The tubes were then centrifuged at 2500 g for 20 min and the supernatants frozen at -20°C. Platelet factor 4 in the supernatants was measured within 24 h by radioimmunoassay (25).

The residual free Ca2+ concentration in the gel filtration buffer was determined using 5 μg/ml of IgG. In studies of the Ca2+ dependence of PAC-1 binding, the free Ca2+ concentration of the incubation mixture was varied from 10^(-9) to 10^(-3) M by the addition of CaCl2 and EGTA (19, 26). The antibody binding data were analyzed on an Apple Macintosh microcomputer by nonlinear regression analysis using the general equation for ligand binding to one or more noninteracting classes of binding sites (27).

**Platelet Function Studies—**Since PAC-1 interacted with platelets only after platelet stimulation, these studies were typically carried out by incubating unstimmed, gel-filtered platelets in an aggregation cuvette at room temperature simultaneously with an agonist and PAC-1. After 5 min, aggregation was initiated by stirring the cuvette in an agitometer at 37°C and adding 200 μg/ml fibrinogen and 100 μg CaCl2. The extent of aggregation and [125I]serotonin release 3 min later was measured (19).

**RESULTS**

Platelet-specific monoclonal antibodies were generated by fusing the murine myeloma cell line, SP-2, with spincic lymphocytes from a mouse that had been immunized with platelets from a patient with Glanzmann's thrombasthenia. These platelets contained approximately 5% of the normal amount of glycoproteins IIb and IIIa as measured by the binding of IIb- and IIIa-specific monoclonal antibodies. Platelet-specific clones were screened further for preferential reactivity with platelets.
“activated” platelets using a solid-phase immunoassay described under “Experimental Procedures.” One of the positive clones, designated PAC-1, produced antibody that consistently reacted with platelets stimulated with ADP, epinephrine, or thrombin but not with unstimulated platelets or platelets incubated with PGI2 (Fig. 1). PAC-1 was purified as described under “Experimental Procedures” and identified as a pentameric, IgM K-immunoglobulin.

PAC-1 Binding to Platelets—Antibody binding studies were performed with radiiodinated PAC-1. 125I-PAC-1 bound minimally to either unstimulated gel-filtered platelets or to platelets incubated with 1 μM PGI2 to prevent platelet activation. In contrast, when normal platelets were activated by the addition of ADP, epinephrine, or thrombin, there was a striking increase in PAC-1 binding. Maximum binding was attained within 15–20 min (Fig. 2A) and was similar at 22 and 37 °C.

The extent of PAC-1 binding to normal platelets was dependent on the agonist concentration. Using ADP or epinephrine, half-maximal binding was observed at an agonist concentration of 0.1 μM. Maximal binding required 1–2 μM (Fig. 2B). In the case of thrombin, 125I-PAC-1 binding was half-maximal at 1–2 milliunits/ml thrombin and maximal at 5 milliunits/ml. ADP-induced PAC-1 binding was prevented by 25 μM ATP, an ADP receptor antagonist. Epinephrine-induced binding was prevented by 1 μM yohimbine, an α2-adrenergic receptor antagonist.

PAC-1 binding to platelets at 22 °C was dependent on the extracellular free Ca2+ concentration. In these studies, the Mg2+ concentration of the buffer was maintained at 1 mM while the free Ca2+ concentration was set at values that ranged from 10–4 to 10–3 M by adding CaCl2 and EGTA (19, 26). Then, ADP and saturating concentrations of antibody were added to the platelet mixture and 125I-PAC-1 binding measured. Little or no PAC-1 binding was observed at free Ca2+ concentrations <10 nM. Half-maximal binding occurred at 4 μM free Ca2+. Maximal binding required at least 1 μM free Ca2+. At free Ca2+ concentrations >100 μM, there was a small, but reproducible, decrease in PAC-1 binding (Fig. 3). Additional studies with membrane proteins solubilized in 0.2% Triton X-100 and 10 mM CHAPS indicated that Ca2+ was required for the exposure of the PAC-1 epitope on platelets rather than for the antigen-antibody reaction per se. Using the ELISA shown in Fig. 1, platelets were first solubilized in the presence of 50 μM free Ca2+, and the proteins were then immobilized on a nitrocellulose membrane. PAC-1 bound to its immobilized antigen, even when the binding reaction was carried out in the presence of 5 mM EGTA.

125I-PAC-1 bound to platelets saturaedly and with a high apparent affinity (Fig. 4). Unstimulated platelets bound approximately 1000 PAC-1 molecules/platelet (Table I). No further increase in PAC-1 binding was seen after incubation periods as long as 2 h. In contrast, platelets stimulated maximally with epinephrine, ADP, or thrombin bound an average of 11,393, 13,348, and 21,107 PAC-1 molecules/platelet, respectively. Only a single class of PAC-1-binding sites was evident, with an apparent KD of approximately 5 nM (Fig. 4 and Table I).

The Relationship of PAC-1 Binding to Platelet Secretion—Two other monoclonal antibodies have been reported to bind preferentially to activated platelets (28, 29). In each case, the antigen involved proved to be a granule membrane protein that appeared on the platelet surface only after secretion. The following studies were performed to determine whether PAC-1 binding is also dependent on platelet secretion. First, PAC-1 binding was compared in platelets prepared with or without aspirin. Inhibition of prostaglandin synthesis by aspirin had no effect on the number of PAC-1 binding sites (Table I).
Second, α-granule and dense granule secretion were measured during the antibody binding assays. Over a broad range of ADP, epinephrine, and thrombin concentrations, there was no relationship between the extent of PAC-1 binding and the release of either [3H]serotonin (Fig. 5) or platelet factor 4 (not shown). These data suggest that the PAC-1 antigen is neither a protein confined to the granule membrane nor a secreted protein.

Identification of the PAC-1-specific Platelet Antigen—In order to identify the antigen on the platelet surface that is recognized by PAC-1, binding studies were also performed with platelets from three donors with homozygous Glanzmann’s thrombasthenia (19). Their platelets contained less than 7% the normal amount of glycoproteins IIb and IIIa. Despite the fact that thrombasthenic platelets had been used for immunizing the mouse that produced PAC-1, PAC-1 binding to ADP-stimulated thrombasthenic platelets was reduced by an average of 94% compared to normal platelets (Fig. 6). This observation suggests that PAC-1 may recognize an epitope on either glycoprotein IIb, IIIa, or the IIb:IIIa complex.
Platelet-rich plasma was incubated with aspirin and \( ^{14}C \)serotonin as described under "Experimental Procedures." After gel filtration, the platelets were incubated for 5 min with either PGI\(_2\) or various concentrations of agonists. \( ^{125}I \)-PAC-1 (10 \( \mu \)g/ml) was then added for 15 min and the extent of PAC-1 binding and \( ^{14}C \)serotonin release measured. Closed circles represent platelets incubated with 0.1-1.0 \( \mu \)M epinephrine; closed squares, 0.05-20 \( \mu \)M ADP; and open squares, 0.5-20 milliunits/ml thrombin. This figure contains data pooled from two experiments.

Furthermore, despite the fact that A2A\(_9\) bound to approximately twice as many sites/platelet as PAC-1, unlabeled A2A\(_9\) did not effectively inhibit PAC-1 binding. A2A\(_9\) was an IgG anti-platelet monoclonal antibody that is specific for the IIb-IIIa complex (8). Unlabeled A2A\(_9\) inhibited \( ^{125}I \)-PAC-1 binding competitively with an apparent \( K_i \) of 7 nM (Fig. 7A). This value is similar to the apparent \( K_i \) for \( ^{125}I \)-A2A\(_9\) binding to platelets (8). Furthermore, despite the fact that A2A\(_9\) bound to approximately twice as many sites/platelet as PAC-1, unlabeled PAC-1 was an effective competitive inhibitor of \( ^{125}I \)-A2A\(_9\) binding. In this case, the apparent \( K_i \) was 9 nM (Fig. 7B). Finally, purified fibrinogen inhibited the binding of \( ^{125}I \)-PAC-1 to ADP-stimulated platelets with an apparent \( K_d \) of 130 nM. This value is similar to reported values for the \( K_d \) of \( ^{125}I \)-fibrinogen binding to ADP-stimulated platelets (1, 2).

Since PAC-1 is an IgM, it was possible that its relatively large size, rather than its specificity, limited its interaction with unstimulated platelets. To test this possibility, we examined the ability of another pentameric IgM monoclonal antibody, A6G8, to bind to platelets. In preliminary studies, A6G8 failed to bind to thrombasthenic platelets and bound exclusively to the IIb-IIIa complex on Western blots. Using the ELISA shown in Fig. 1, we found that unlike PAC-1, A6G8 bound equally well to both unstimulated and stimulated platelets. Thus, it is unlikely that the large molecular size of PAC-1, per se, can explain its preferential binding to stimulated proteins.

As further evidence that the IIb-IIIa complex is the antigen recognized by PAC-1, we preincubated platelet-rich plasma with 5 mM EGTA for 1 h at 37°C. These conditions were shown previously to dissociate the IIb-IIIa complex irreversibly (12). Afterwards, 5 mM CaCl\(_2\) was added, the platelets were gel-filtered, and ADP-stimulated PAC-1 binding was measured. In two experiments, \( ^{125}I \)-PAC-1 binding to control platelets that had been preincubated at 37°C without EGTA or at 22°C with EGTA was normal. Under both of these conditions, the IIb-IIIa complex remains intact (12). In contrast, the binding of PAC-1 to platelets preincubated with EGTA at 37°C was decreased by 84%.

Finally, PAC-1 was shown to bind directly to the IIb-IIIa complex on Western blots. Platelets were solubilized with a mixture of Triton X-100 and CHAPS, either in the presence of 1 mM Ca\(^{2+}\) to maintain the IIb-IIIa complex or with 5 mM EDTA to dissociate it. The extracts were electrophoresed in a nondenaturing polyacrylamide gradient gel system that is capable of separating the IIb-IIIa complex from the disso-
cated glycoproteins (12). Western blots of these gels were then incubated with PAC-1 in the presence of 10 μM Ca²⁺. Bound antibody was detected using peroxidated-conjugated goat anti-mouse immunoglobulin. PAC-1 bound to the intact IIb-IIIa complex (Fig. 8). However, the antibody did not bind to the dissociated forms of IIb or IIIa.

**Effect of PAC-1 on Platelet Function**—In the absence of an agonist, PAC-1 at concentrations as high as 50 μg/ml did not cause platelet aggregation, [¹⁴C]serotonin release, or the release of platelet factor 4. PAC-1 also had no effect on ADP-induced platelet shape change, but it did inhibit ADP- and epinephrine-induced platelet aggregation and serotonin release. This effect was most pronounced if the agonist and the PAC-1 were incubated with the platelets for 5 min before initiating the aggregation reaction by adding fibrinogen and stirring. Under these conditions, platelet aggregation was inhibited half-maximally by 5-10 nM PAC-1, a value similar to the apparent Kₐ for [¹²⁵I]PAC-1 binding to stimulated platelets (Fig. 9). Similar results were obtained for inhibition of platelet [¹⁴C]serotonin release. Thus, PAC-1 inhibited fibrinogen-mediated platelet aggregation, and conversely, fibrinogen inhibited [¹²⁵I]-PAC-1 binding. This suggests that PAC-1 inhibited platelet aggregation by preventing the interaction of fibrinogen with its receptor.

**DISCUSSION**

One of the required steps in platelet aggregation is the expression of receptors for fibrinogen on the platelet surface. Although this receptor is known to be located on the glycoprotein IIb-IIIa complex, the changes involving the complex that result in exposure of the binding site for fibrinogen are unknown. Receptor expression does not appear to be the outcome of simple formation of the complex from previously separate components because IIb-IIIa heterodimers are present in the membrane of unstimulated platelets (7-12). In the present studies, we have described a pentameric IgM monoclonal antibody, PAC-1, that binds to the surface of platelets activated by agonists such as ADP, epinephrine, and thrombin, but does not bind to resting platelets. Unlike two previously reported monoclonal antibodies that bind preferentially to activated platelets (28, 29), PAC-1 binding is not dependent upon platelet secretion.

Western blots of nondenatured platelet proteins were used to identify the glycoprotein IIb-IIIa complex as the PAC-1-specific antigen. In addition, PAC-1 has been shown to bind to the complexed but not the dissociated form of IIb and IIIa on crossed immuno-electrophoresis.² Several other lines of evidence also indicated that PAC-1 binds to the IIb-IIIa complex. First, PAC-1 bound to activated normal platelets, but failed to bind to platelets from three patients with Glanzmann's thrombasthenia. It should be noted that thrombathenic platelets, containing about 5% the normal amount of IIb and IIIa, had been used to immunize the mouse that produced PAC-1. The fact that an anti-IIb-IIIa monoclonal

² T. Kunicki, personal communication.
antibody was obtained emphasizes the immunogenicity of this glycoprotein complex. Second, A2A is an IgG monoclonal antibody previously shown to be specific for the IIb-IIIa complex, competitively inhibited 125I-PAC-1 binding. Conversely, PAC-1 competitively inhibited 125I-A2Ag binding. Third, in the epitope on the platelet surface. However, this alternative activated platelets was Ca2+-dependent. Half-maximal dissociation membrane IIb from IIIa (12), also resulted in the loss of PAC-1 binding. Fourth, the binding of PAC-1 to activated platelets was Ca2+-dependent. Half-maximal binding of PAC-1 occurred at 0.4 μM Ca2+, a concentration that is identical to the Kd for Ca2+ of a class of Ca2+-binding sites associated with the IIb-IIIa complex (19).

The epitope on the IIb-IIIa complex to which PAC-1 binds appears to be close to the fibrinogen receptor site. When PAC-1 was added to activated, gel-filtered platelets before the addition of fibrinogen, the antibody inhibited aggregation, a platelet response that requires fibrinogen binding. Conversely, fibrinogen inhibited the binding of 125I-PAC-1 to platelets in a dose-dependent manner. These data suggest that PAC-1 recognizes an epitope on IIb-IIIa that becomes accessible to the antibody and to fibrinogen only after stimulation of the platelet with an agonist. The accessibility of this epitope upon platelet activation provides direct evidence for the hypothesis that fibrinogen receptor expression actually represents an agonist-induced change in the IIb-IIIa complex.

Depending upon the agonist, platelets bound 10,000–25,000 PAC-1 molecules/platelet. In contrast, studies with radioiodinated fibrinogen and A2Ag show that there are an average of 40,000–50,000 copies of the IIb-IIIa complex/fibrinogen receptor on the platelet surface (1, 8). Although there are several possible explanations for this discrepancy between the PAC-1 and the A2A or fibrinogen binding data, the most likely is that the pentameric structure of PAC-1 allows it to bind to more than one site at the same time. Studies which show that IIb-IIIa complexes may cluster during platelet activation are consistent with this possibility (30). Alternatively, PAC-1 may recognize only a subset of the total number of IIb-IIIa complexes. However, the observation that A2A and PAC-1 are mutual competitive inhibitors would seem to exclude this latter possibility.

Thrombin-activated platelets had significantly more PAC-1-binding sites than did platelets stimulated with ADP or epinephrine. It has been suggested that in resting platelets, IIb-IIIa is associated not only with surface membranes but with granule membranes and surface-connecting membranes (31, 32). The greater number of PAC-1-binding sites on thrombin-stimulated platelets may reflect exposure of these otherwise inaccessible IIb-IIIa complexes.

Extracellular Ca2+ plays a multifactorial role in the function and stability of the IIb-IIIa complex. First, in the absence of Mg2+, 0.1–1.0 mM Ca2+ is required for fibrinogen binding. This function of Ca2+ can also be served by Mg2+ (1). Second, studies with both intact platelets and with the detergent-solubilized glycoproteins have demonstrated that the IIb-IIIa heterodimer is held together by Ca2+ (11, 12, 14–17). In this case, the Kd for 0.4 μM and Mg2+ cannot substitute for Ca2+ (33). Third, as demonstrated in the present studies, the Ca2+-dependence of PAC-1 binding at 22 °C suggests that Ca2+ must be present for agonist-induced expression of the fibrinogen receptor. The Kd for this reaction was also approximately 0.4 μM and Mg2+ could not substitute for Ca2+. It is difficult to completely exclude the possibility that Ca2+ is required for antibody function and not for the expression of the epitope on the platelet surface. However, this alternative seems less likely because PAC-1 bound to solubilized IIb-IIIa complexes on nitrocellulose membranes equally well in the presence and absence of Ca2+.

In contrast to PAC-1 binding, A2A binding to intact platelets is Ca2+-dependent at 37 °C, but not at 22 °C. The loss of A2A binding at the higher temperature is readily explained by the fact that Ca2+ depletion at 37 °C, but not at 22 °C, causes dissociation of the IIb-IIIa complex within the membrane (12). The difference between PAC-1 binding and A2A binding at 22 °C suggests that removing Ca2+ at this lower temperature does not dissociate the complex but does affect the ability of IIb-IIIa to undergo the changes that expose the fibrinogen receptor. That Ca2+ may be required for the receptor to undergo a conformational transition that then enables it to bind fibrinogen. Ca2+-dependent structural transitions have been described for coagulation proteins such as prothrombin and factors IX and X (34–37). In these cases, the changes in protein conformation have a major impact on the function of the proteins.

Three other anti-platelet monoclonal antibodies that react preferentially with stimulated platelets have been described recently. Two of these, KC4 and S12, recognize a M, 140,000 antigen that appears on the platelet surface only after platelet secretion (28, 29). This antigen normally resides on the intracellular membranes of α-granules. Stimulation by agonists causes a secretory response that is accompanied by the fusion of granule membranes to plasma membranes resulting in exposure of the antigen on the platelet surface. In contrast to these antibodies, PAC-1 binding was not dependent upon platelet secretion and was specific for the IIb-IIIa complex.

The third activation-specific antibody, 7E3, has been described in preliminary form by Coller (38). Like PAC-1, 7E3 appeared to bind to the IIb-IIIa complex. In contrast to PAC-1, however, maximum binding of 7E3 to resting platelets was similar to that of activated platelets. However, platelet activation caused an increase in the rate of binding of the antibody. It was concluded from this observation that 7E3 recognizes a change in either the conformation or the microenvironment of IIb-IIIa that is enhanced by agonists, but occurs slowly even in the absence of an agonist. The precise explanation for the differential rate of binding of 7E3 to activated platelets and the relationship of the epitope for this antibody to the one recognized by PAC-1 remain to be determined.

Monoclonal antibodies specific for platelets offer great potential for improvements in the diagnosis of thrombotic disorders. For example, preliminary experiments in animals have demonstrated the feasibility of linking these antibodies to indium-111 for purposes of imaging arterial and venous thrombi (39). In addition, the secretion-dependent antibody, S12, has been used in direct binding studies on whole blood to detect activated platelets in some, but not all, patients with acute respiratory distress syndrome. Since PAC-1 reports on a platelet surface change that is more subtle than secretion, this antibody may prove to be a sensitive reagent to detect the presence of activated platelets in thrombotic disorders in man.

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3 J. George and R. McEver, personal communication.
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