On the Association of Glycoprotein Ib and Actin-binding Protein in Human Platelets

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ABSTRACT Glycoprotein (GP) Ib was purified from lysates of human platelets prepared in the presence or absence of inhibitors of the endogenous calcium-activated neutral protease (CANP) by immunoaffinity chromatography, employing the GPIb-specific murine monoclonal antibody, AP1, coupled to Sepharose CL4B. When derived from lysates prepared in the presence of EDTA or leupeptin, the eluate from the AP1-affinity column contained a 240,000–260,000-mol-wt protein in addition to GPIb. In SDS PAGE, this protein was stained by Coomassie Blue R, but not by the periodic acid-Schiff reagent, and it was not labeled with 125I in intact platelets by the lactoperoxidase-catalyzed method. When derived from lysates prepared in the absence of CANP inhibitors, the eluate contained only GPIb and its proteolytic derivative, glyocalicin.

A change in the electrophoretic mobility of GPIb consistent with its association with the 240,000–260,000-mol-wt protein was confirmed by crossed immunoelectrophoresis. By an immunoblot technique involving transfer of proteins eluted from the AP1-affinity column and separated by SDS PAGE onto a nitrocellulose membrane, the 240,000–260,000-mol-wt protein bound polyclonal goat antibody raised against rabbit macrophage actin-binding protein (ABP).

On the basis of these results, we conclude the GPIb is tightly associated with ABP under conditions in which the endogenous CANP is inhibited, and that this apparent transmembrane complex of GPIb-ABP can be isolated in lysates of nonactivated human platelets.

The murine monoclonal antibody, AP1, recognizes an epitope common to human platelet membrane glycoprotein (GP) Ib and its proteolytic derivative, glyocalicin (9, 11, 20). In a rapid whole blood binding assay (11), AP1 was shown to react with platelets from all normal donors tested and six patients with Glanzmann’s thrombasthenia, a hereditary disorder of platelet cohesion characterized by the absence of GPIIb and GPIIIa (13). By the same assay, no reaction was observed with platelets from three patients with the Bernard-Soulier syndrome (BSS), a hereditary disorder of platelet-vessel wall adhesion characterized by the absence of platelet GPIb (13). Recently, Ruggeri et al. (20) showed that platelet binding of [125I]von Willebrand factor (vWF) induced by ristocetin was completely blocked by prior exposure to AP1, while that induced by ADP + epinephrine was unaffected. This finding supports the hypothesis that GPIb is involved in the ristocetin-induced binding of vWF, while the GPIIb–GPIIIa complex, but not GPIb, is involved in the expression of thrombin- or ADP + epinephrine-induced platelet receptors for vWF.

To obtain additional insight into the structure and function of GPIb and its relationship to other membrane and nonmembrane components, we purified the glycoprotein by immunoaffinity chromatography. Since Solum et al. (23) had reported that GPIb may be associated in complexes that are disrupted by the endogenous calcium-activated neutral protease (CANP), we analyzed lysates prepared in the presence or absence of inhibitors of CANP. As noted in a preliminary report (9), this experimental approach confirmed the existence of soluble complexes of GPIb and a 240,000–260,000-mol-wt platelet protein. We now report the identification of this protein as actin-binding protein (ABP).

MATERIALS AND METHODS

Monoclonal IgG was isolated from ascites fluids and labeled with 125I as previously described (11, 17). Crossed immunoelectrophoresis (CIE) was per-
formed as previously described, incorporating labeled monoclonal IgG into the intermediate gel (8, 11), and precipitin arcs containing bound monoclonal antibody were revealed by autoradiography. Immunoaffinity chromatography using AP1 coupled to Sepharose CL4B (Pharmacia Fine Chemicals, Piscataway, NJ) and assays of the direct binding of [125I]AP1 to platelets were performed as described (17). SDS PAGE was performed in 3-mm thick, 7% acrylamide slab gels as described by Norden et al. (14). Apparent molecular weights were calculated by direct comparison to the mobilities of the following known protein standards (Bio-Rad Laboratories, Richmond, CA): myosin (200,000); beta-galactosidase (117,000); phosphorylase b (94,000); bovine serum albumin (69,000); and ovalbumin (43,000).

**Immunoblot Method:** After SDS PAGE, gels were incubated in 0.025 M Tris-HCl, 0.192 M glycine, 20% (vol/vol) methanol (pH 8.2) for 30 min at ambient temperatures and sandwiched against a nitrocellulose membrane (Bio-Rad Laboratories) in a Transblot apparatus (Bio-Rad). Proteins were transferred by electrophoresis at 60 V for 3 h and 30 min at 4°C. Nonreacted nitrocellulose was then blocked by incubation for 1 h in 0.01 M Tris-HCl, 0.145 M NaCl (pH 7.0) containing 1% gelatin, followed by two 10-min washes in the same buffer containing 0.5% gelatin, 0.5% Triton X-100 and 0.01% NaN3. The membrane was then incubated for 18 h with agitation in 50 ml of the latter mixture containing 0.25 ml of goat antiserum raised against rabbit macrophage ABP. This antiserum, a generous gift from John H. Hartwig, Ph.D. (Hematology-Oncology Unit, Massachusetts General Hospital, Boston) cross-reacts with ABP from human platelets. The membrane was then washed five times in the same buffer mixture without antiserum, incubated for 6 h in 50 ml of the same buffer mixture containing affinity-purified, [125I]-rabbit anti-goat IgG (106 cpm/ml [Zymed Labs, Inc., So. San Francisco, CA]), and finally washed five times in the same buffer mixture containing 0.2% SDS. The membrane was immediately blotted without drying and packaged within a plastic bag. For autoradiography, membranes within plastic bags were sandwiched between Kodak XRP-1 x-ray film and a Dupont Cronex Hi Plus Intensifying screen for 1 to 24 h at ambient temperature in a light-proof Kodak x-ray film cassette.

Additional murine monoclonal antibodies were used in these studies: antifibronectin (ATCC CRL 1605) was obtained commercially; anti-vWF (AVW2), reactive with all multimers, was produced in the laboratory of Dr. Robert Montgomery (21). Platelet cytoskeletons, as a source of semi-purified ABP, were prepared as described by Rosenberg et al. (19), except that 0.01 M HEPES was used in place of 0.01 M imidazole-chloride in the “Triton solubilization buffer.” This modification was without apparent effect upon the composition of the cytoskeleton complex subsequently isolated.

**RESULTS**

**Steady-state Binding of AP1 to Human Platelets**

On the basis of data derived from eight different healthy donors, we found that the number of AP1 molecules bound per platelet at saturation was 34,200 ± 5,400 (mean ± SD), with an average dissociation constant (Kd) of 2.1 ± 0.4 nM. No difference in binding was noted at room temperature in the presence or absence of divalent cations. Comparative studies with washed platelets derived from whole blood anti-coagulated with citrate, EDTA, acid-citrate-dextrose, or heparin gave equivalent results.

**CIE**

[125I]AP1 was incorporated into the intermediate gel before the start of the second dimension electrophoresis, and the precipitin arcs containing antigens reactive with AP1 were revealed by autoradiography of the dried gels (Fig. 1). When platelets were lysed in the absence of leupeptin or EDTA, AP1 bound solely to two cross-reactive precipitin arcs known from previous studies (6) to contain GPIb and glycocalcin, respectively (Fig. 1 B). In lysates from identical platelets prepared in the presence of 0.4 mM leupeptin (Fig. 1 A), at least two additional cathodic precipitin arcs were labeled by [125I]-AP1, one of which was a sharp “spike” situated directly over the sample well (open arrowhead in Fig. 1A). Essentially the same results were obtained from lysates prepared in the presence of 5 mM EDTA, although the spike precipitin arc was not as pronounced as that seen in lysates prepared with leupeptin. Radiolabeled, monoclonal antifibronectin and anti-vWF (AVW2) bound to respective precipitin arcs known to contain these antigens, but neither bound to the cathodic precipitin arcs reactive with radiolabeled AP1 (data not shown).

**Immuoaffinity Chromatography**

Purified AP1 IgG was covalently coupled to cyanogen bromide-activated Sepharose CL4B. Triton X-100 lysates derived from the same preparation of washed, [125I]-labeled platelets, in the absence or presence of 0.4 mM leupeptin, were chromatographed on identical AP1-Sepharose columns equilibrated in buffer containing leupeptin. Bound protein was eluted by a multistep elution protocol (17); the final
elution buffer contained 0.05 M diethylamine (pH 11.5). Each eluate was then analyzed by SDS PAGE. Protein bands were visualized by staining with Comassie Blue-R (CBR) or periodic acid-Schiff reagent (PAS).

When lysates prepared in the presence of leupeptin were analyzed, a PAS-positive, radiolabeled protein with an electrophoretic mobility following reduction with 2-mercaptoethanol characteristic of the larger subunit, or alpha chain, (145,000-mol-wt), of GPIb was eluted only upon addition of diethylamine (Fig. 2, solid arrowhead in lanes A–C). A PAS-positive band of identical mobility and present in the original lysate (Fig. 2, lane I) was seen to be depleted from the nonadherent fraction (Fig. 2, lane 2). In addition to GPIb alpha, the diethylamine-eluate contained a high molecular weight protein (240,000–260,000) that was stained by CBR (Fig. 2, open arrowhead in lane A), but PAS-negative and nonradiolabeled (Fig. 2, lanes B and C). Eluates derived by addition of 1 M NaCl often contained detectable amounts of the 240,000–260,000-mol-wt protein, but never contained a protein band equivalent to GPIb alpha. Identical proteins were contained in diethylamine-eluates derived from lysates prepared in the presence of 5 mM EDTA.

When lysates prepared in the absence of leupeptin or EDTA were analyzed, diethylamine-eluates were found to contain two closely migrating protein bands (with molecular weights of 140,000 and 135,000) that were both PAS-positive and radiolabeled (Fig. 2, arrows in lane D). Based upon these apparent molecular weights, the former protein is likely GPIb; the latter, glycocalicin. No trace of the 240,000–260,000-mol-wt protein was detected either in diethylamine-eluates or 1 M NaCl-eluates derived from lysates prepared in the absence of leupeptin or EDTA.

**Immunoblot**

As shown in Fig. 3, the platelet lysate used to generate purified GPIb (lane A) was directly compared to a cytoskeleton complex preparation enriched in ABP (lane B), and the fraction containing the GPIb-high molecular weight protein complex eluted from an AP1-Sepharose affinity column (lane C). By this method, the 240–260,000-mol-wt protein that is co-purified with GPIb (cf. Fig. 2, lane A) was shown to cross-react with antibody specific for ABP.

**Specificity of AP1**

As shown previously, AP1 reacts with GPIb and glycocalicin (9, 11, 20). In the present study, it was necessary to show that AP1 does not also react with ABP leading to artifactual isolation of ABP along with GPIb. The monoclonal antibody, AP1, is nonreactive in the SDS PAGE immunoblot technique. Thus, it became necessary to isolate ABP from GPIb. As it was difficult to prepare an ABP preparation that does not contain some contamination of GPIb, we used platelets from a patient with the BSS which lack GPIb (13). A cytoskeletal preparation from BSS platelets contains ABP as demonstrated by immunoblot technique with goat anti-ABP serum. Reaction of AP1 with the cytoskeletal preparation from BSS platelets could not be detected using an ELISA technique. The cytoskeletal preparation was adsorbed onto Immulon 1 plates (Dynatech Laboratories, Inc., Alexandria, VA) and reaction with AP1 assayed using the biotin/avidin system for mouse IgG detection (Vector Laboratories, Inc., Burlingame, CA).

**DISCUSSION**

Based upon partial immunologic identity and similarities in tryptic peptide maps, there is now little doubt that GPIb and glycocalicin are structurally related (1, 15, 22), and that glycocalicin is derived from GPIb by the action of an endogenous CANP (10, 22, 23, 25).

Using CIE, Solum et al. (23) demonstrated that cleavage of GPIb by CANP can occur within intact platelets stimulated with local anesthetics, such as dibucaine, and that the mobility of GPIb within the first dimension of CIE gels is impeded in lysates prepared under conditions that would inhibit the endogenous CANP. Activation of the endogenous CANP in intact platelets with the ionophore A23187 in the presence of calcium has also been shown to result in the proteolysis of GPIb with release of glycocalicin (10). An additional substrate of CANP, ABP, has also been shown to be cleaved concomitant with platelet activation by thrombin or A23187 (7, 16, 24). Thus, it has been postulated that proteolysis of selected substrates, perhaps GPIb and/or ABP, by CANP may be a requisite step in platelet activation.

The results of our studies confirm and extend the initial
that the association described by Fox et al. (6) is identical to the Triton-insoluble cytoskeleton as described by Fox et al. (6) would suggest that a transmembrane linkage exists between GPIb and ABP in the intact platelet. The potential physiologic relevance of such an interaction is compelling, and one can imagine that this transmembrane interaction could limit the mobility of both components in nonactivated platelets until the linkage is destroyed by activation of CANP. However, the possibility that this association only occurs subsequent to platelet disruption must be considered, and a more rigorous confirmation of this hypothesis would entail analyses of the association(s) of GPIb within intact platelets.

Aside from its association with a 17,000–22,000-mol-wt minor glycoprotein, known as GPIIX or GPI17 (2, 4, 14), the use of immunoelectron microscopy (18) or chemical cross-linking reagents (5) has not revealed any significant interaction between GPIb and other platelet proteins. However, the failure to observe such interactions with the latter method (5) may be related to the relative difficulty in detecting GPIb with protein-specific stains or by surface-radioiodination of platelets.

Given that a transmembrane linkage between GPIb and the cytoskeleton does exist in intact platelets, one might expect that agents that would perturb this interaction could also influence GPIb receptor function. In this regard, Coller (3) has recently reported that tertiary amine local anesthetics initially cause an increase in the rate of vWF-dependent platelet aggregation, whereas prolonged exposure to these agents produces a significant inhibition of vWF-dependent platelet agglutination that is temporally correlated with proteolysis of GPIb. Since such local anesthetics are also known to rapidly induce reversible platelet-sphering and retraction of pseudopodia in the absence of proteolysis (12), it is conceivable that the enhanced vWF-dependent aggregation observed by Coller (3) is due to an increased mobility of vWF receptors (GPIb) caused by disruption of the GPIb-cytoskeleton interaction. Prolonged exposure to these agents sufficient to cause proteolysis of GPIb and/or ABP results in irreversible inhibition of vWF-dependent agglutination (3) and irreversible platelet-sphering (12). Studies are now in progress to analyze in greater detail the effect of GPIb-ABP complex integrity upon platelet functions attributed to GPIb.

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