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

JANICE R. OKITA, DOMINIQUE PIDARD, PETER J. NEWMAN, ROBERT R. MONTGOMERY, and THOMAS J. KUNICKI

The Blood Center of Southeastern Wisconsin, Inc., Milwaukee, Wisconsin 53233; and Department of Microbiology and Pediatrics of the Medical College of Wisconsin, Milwaukee, Wisconsin 53226

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 ^{125}I 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, glycocalicin.

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)[1] Ib and its proteolytic derivative, glycocalicin (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 [^{125}I]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 ^{125}I as previously described (11, 17). Crossed immunoelectrophoresis (CIE) was per-

[1] *Abbreviations used in this paper:* ABP, actin-binding protein; BSS, Bernard-Soulier syndrome; CANP, calcium-activated neutral protease; CBR, Coomassie Blue-R; CIE, crossed immunoelectrophoresis; GP, glycoprotein; PAS, periodic acid-Schiff reagent; vWf, von Willebrand factor.

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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 [^{125}I]AP1 to platelets were performed as described (17). SDS PAGE was performed in 3-mm thick, 7% acrylamide slab gels as described by Nurden 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% NaN_3. 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[2]. 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, [^{125}I]-rabbit anti-goat IgG (10^6 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: anti-fibronectin (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 Rosenburg 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 (K_d) 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 anticoagulated with citrate, EDTA, acid-citrate-dextrose, or heparin gave equivalent results.

CIE

[^{125}I]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 glycocalicin, 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 [^{125}I]-AP1, one of which was a sharp "spike" situated directly over

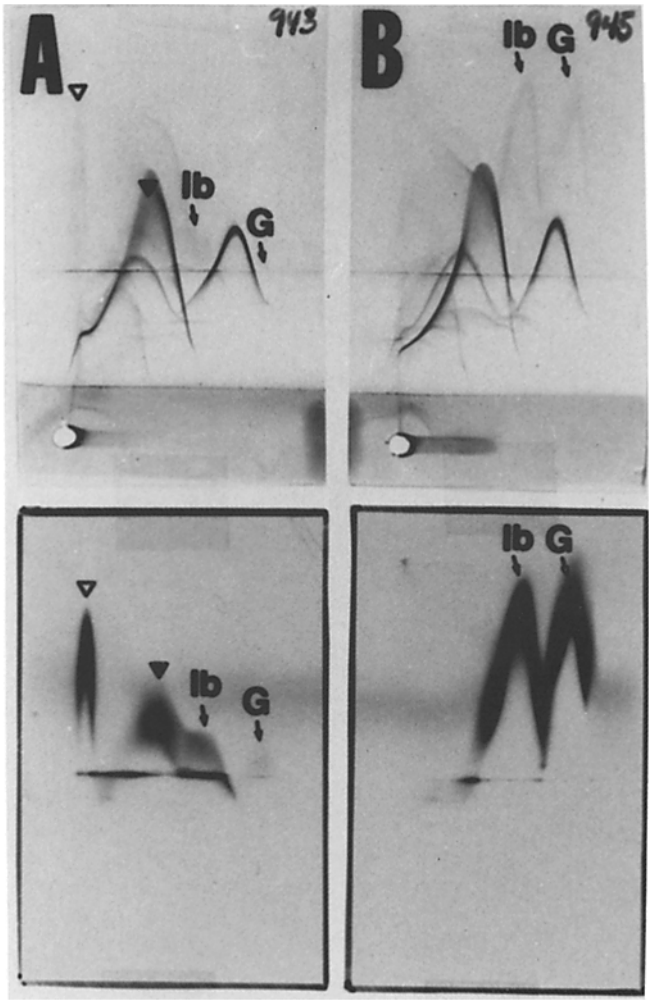

FIGURE 1 CIE. Identical washed platelet samples were lysed in the presence (*A*) or absence (*B*) of 0.4 mM leupeptin. Triton X-100 soluble protein (100 μg) was electrophoresed against a mixture of polyspecific rabbit anti-human platelet antibody and [^{125}I]AP1. Gels were stained with CBR, dried, and subjected to autoradiography. The resultant autoradiographs were depicted below the corresponding CBR-stained gel. The position of the precipitin arcs given by GPIb and glycocalicin (*G*) are indicated. Arrowheads in gel *A* indicate the additional cathodic precipitin arcs to which [^{125}I]AP1 also bound.

the sample well (open arrowhead in Fig. 1*A*). 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).

Immunoaffinity Chromatography

Purified AP1 IgG was covalently coupled to cyanogen bromide–activated Sepharose CL4B. Triton X-100 lysates derived from the same preparation of washed, ^{125}I-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

[2] J. Hartwig, personal communication.

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 Commassie 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 *1*) 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 has been 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 I 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).

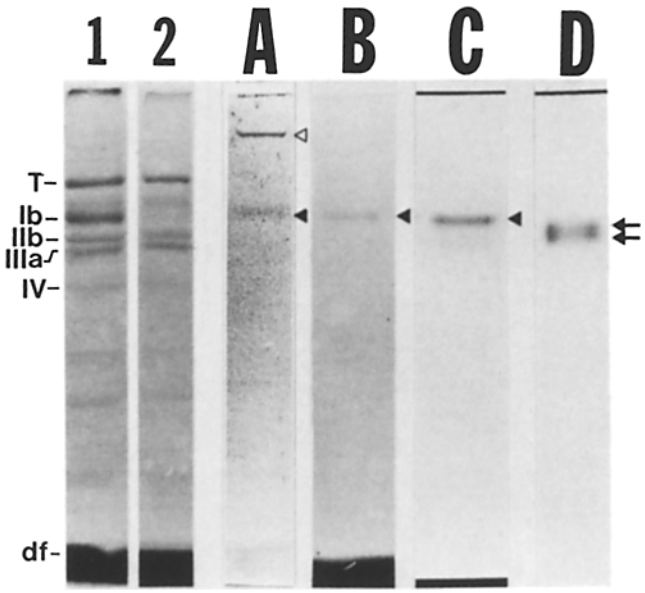

FIGURE 2 Immunoaffinity chromatography. Lysates from ^{125}I-labeled platelets prepared in the presence or absence of leupeptin were passed through affinity columns composed of AP1 coupled to Sepharose CL4B. Protein composition of column fractions was determined by SDS PAGE after addition of 2-mercaptoethanol. Indicated in the figure: the total platelet lysate stained with PAS (lane *1*); the nonadherent fraction stained with PAS (lane *2*); bound protein eluted with diethylamine, stained with CBR (lane *A*) or PAS (lane *B*), or visualized by autoradiography (lanes *C* and *D*). Protein in lanes *1*, *2*, *A*, *B*, and *C* was derived from platelets lysed in the presence of leupeptin; protein in lane *D* was derived from the same platelet sample lysed in the absence of leupeptin. The positions of selected platelet proteins (thrombospondin [*T*], and glycoproteins *Ib* alpha, *IIb* alpha, *IIIa*, and *IV*) are indicated in lane *1*. Open arrowhead in lane *A* indicates the position of the protein that is co-purified with GPIb. The apparent molecular weight of this protein is estimated to be in the range of 240,000–260,000 by extrapolation from the highest molecular weight standard used (myosin [M_r = 200,000]). Closed arrowheads in lanes *A–C* indicate the position of glycoprotein Ib alpha (M_r 145,000). Double arrows in lane *D* indicate the position of glycoprotein Ib alpha (M_r 140,000) and glycocalicin (M_r 135,000). *df*, Dye front.

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

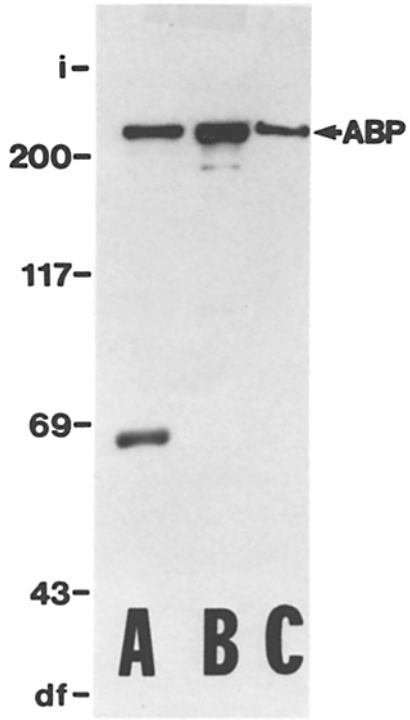

FIGURE 3 Immunoblot. Platelet proteins contained in (*A*) a platelet lysate prepared in the presence of 5 mM EDTA, (*B*) isolated platelet cytoskeleton complex, and (*C*) the eluate from an AP1-Sepharose column derived from the lysate shown in *A*, were separated by SDS PAGE and transferred to nitrocellulose. Bound proteins were then exposed to goat anti-rabbit macrophage ABP followed by [^{125}I]-rabbit anti-goat IgG. The autoradiograph derived from this immunoblot experiment is depicted in this figure. The intensely labeled band corresponding to ABP is indicated. In *A*, an additional labeled band is present that co-migrates with human serum albumin in reduced (M_r 65,000–67,000) or nonreduced (M_r 45,000–50,000) (not shown) SDS PAGE/immunoblot, and thus is probably platelet albumin. Human serum albumin also reacts with the anti-ABP serum in these immunoblot experiments (data not shown). The position of molecular weight markers is indicated to the left of the figure ($M_r \times 10^{-3}$). *i*, Interface between stacking gel and resolving gel; *df*, dye front.

observation of Solum et al. (23), demonstrating the existence of complexes of GPIb and a 240,000–260,000-mol-wt protein. The identification of this protein as ABP is based upon the following facts: (*a*) it is CBR-positive but PAS-negative and thus not likely a glycoprotein (Fig. 2); (*b*) it is not surface-labeled with ^{125}I by the lactoperoxidase method (Fig. 2) or with ^{3}H by periodate oxidation and reduction with sodium borotritide[3]; (*c*) it has an electrophoretic mobility in SDS PAGE identical to that of ABP (Fig. 3); and, most important, (*d*) it cross-reacts with antibodies specific for ABP (Fig. 3). Our data do not permit us to conclude that there is a direct linkage between GPIb and ABP, and the possibility that this association is mediated by additional platelet proteins remains to be investigated. In a preliminary communication, Fox et al. (6) reported that GPIb is linked to a 250,000-mol-wt polypeptide in platelet cytoskeletons, but the protein in question was not identified. In view of our findings, it is likely that the association described by Fox et al. (6) is identical to the GPIb-ABP interaction reported herein.

The finding of GPIb-ABP complexes in platelet lysates as described in this report or of a linkage between tritiated GPIb and 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 GPIX or GP17 (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 progess to analyze in greater detail the effect of GPIb-ABP complex integrity upon platelet functions attributed to GPIb.

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