Identification and Isolation of a Platelet GPIb-like Protein in Human Umbilical Vein Endothelial Cells and Bovine Aortic Smooth Muscle Cells

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Abstract

Glycoprotein Ib (GPIb) is an intrinsic platelet membrane protein that plays a major role in platelet adherence and mediates ristocetin-dependent platelet von Willebrand factor binding. Recent reports that the platelet membrane glycoprotein complex IIIb/IIIa is expressed in several cell types prompted us to look for GPIb expression in other vascular cells. Immunoperoxidase staining of human stomach muscle cell layers. Western blotting using monospecific polyclonal anti-GPIb antibodies confirmed the presence of immunoreactive GPIb in human umbilical vein endothelial and bovine aortic smooth muscle cell cultures. Fab fragments of a monospecific anti-GPIb antibody were used to immunoprecipitate [3H]leucine labeled GPIb from metabolically labeled cells. The GPIb in these cells was functional as measured by ristocetin-dependent cell agglutination and by vWF binding. Endothelial cells as well as smooth muscle cells bound 125I-labeled vWF in a ristocetin-dependent manner, with a Kᵦ of 7.9 nM.

Introduction

Glycoprotein Ib (GPIb) is a 165-kD intrinsic platelet membrane protein composed of disulfide linked alpha (145 kD) and beta (22 kD) chains (1). Glycoprotein Ib is a platelet receptor for the von Willebrand factor (vWF) and plays a major role in mediating platelet adhesion to the subendothelium (2). In resting platelets, the membrane glycoprotein is linked to the cytoskeleton (3–5).

Recently, the glycoprotein IIIb/IIIa complex, another intrinsic platelet receptor complex, which mediates platelet aggregation, has been described in cells other than platelets. GPIIb/IIIa-like molecules are present in endothelial cells, smooth muscle cells and fibroblasts (6–8). The broad tissue distribution of IIIb/IIIa and the emerging homologies between adhesive protein receptors of various tissues prompted us to explore the possibility that GPIb might be similarly broadly distributed.

Methods

Materials. Sephacryl S-1000, Sepharose 4B, and PD 10 column (Sephadex G-25 M) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Na25I was purchased from New England Nuclear, Boston, MA. Human plasma was obtained from the New York Blood Center. Fetal calf serum was purchased from Flow Laboratories (McLean, VA), tissue culture flasks from Corning Glassware, Inc. (Corning, NY), fungizone from E. R. Squibb & Sons, Inc. (Princeton, NJ), ristocetin sulfate from Lenau and Co. (Copenhagen, Denmark), calf skin gelatin from Eastman, sodium meta-bisulphite and EDTA from J. T. Baker Chemical Co. (Phillipsburg, NJ), and endothelial cell growth factor (ECGF) from Meloy Laboratories, Inc. (Springfield, VA). Medium 199, modified Eagle's medium, l-glutamine, penicillin-streptomycin solution, and trypsin-verseine mixture were purchased from Whittaker M. A. Bioproducts (Walkersville, MD). Heps, bovine serum albumin (BSA, 35% solution), heparin, gelatin, chloramine-T, polyethylene glycol (PEG), and antibacterial antiserum were purchased from Sigma Chemical Co., St. Louis, MO.

Antibodies. Glycocalcin was prepared as previously described. A monospecific polyclonal anti GPIb was prepared by immunizing rabbits with glycocalcin, a calf plasma product of the alpha chain of GPIb. The monoclonal antibody 3G6 was prepared by standard techniques (9) from mice immunized with purified glycocalcin. Both polyclonal and monospecific antibodies demonstrated GPIb alpha specificity in whole platelet membrane Western blots. The polyclonal antibody blocked ristocetin-induced platelet agglutination in platelet-rich plasma without affecting ADP-, collagen-, thrombin-, or epinephrine-induced platelet aggregation. 3G6 monoclonal did not inhibit ristocetin-induced platelet aggregation. For some studies, AP1, a monoclonal antibody that inhibits ristocetin-induced platelet aggregation (kindly furnished by Dr. T. Kunicki, Milwaukee Blood Center), was used (10). For some studies a second monospecific-polyclonal antibody prepared to reduced-alkylated GPIb-alpha was used (kindly furnished by Dr. Z. Ruggeri, Scripps Institute, La Jolla, CA) (11). vWF purification. vWF was purified by a modification of the procedure of Michelson et al. (12). 10 U of cryoprecipitate obtained from the New York Blood Center was resuspended in 20 mM Na citrate and the pH was adjusted to 6.1 with 20 mM citric acid. 40% PEG (wt/vol) was added to give a final concentration of 2% and the resulting precipitate discarded. Additional PEG was added to a final concentration of 12% and the precipitate collected by centrifugation at 5,000 g for 20 min. The pellet was washed once with 10 ml of ice-cold 0.05 M Tris, 0.15 M NaCl, 8% ethanol, pH 7.8 in order to remove residual PEG, and washed again with 10 ml of 50 mM Tris 0.15 M NaCl, and 0.02% NaN3, pH 7.8 (TBS). The pellet was warmed to 37°C, suspended in TBS, and applied to a 2.5 × 100 cm siliconeized glass column containing Sephacryl S-1000 after removing insoluble materials by centrifugation. 4-ml fractions were collected at a 40-80-ml/h flow rate and analyzed for protein concentration by following absorbance 280 nm. Immunoreactivity of the fractions with monospecific anti-vWF antibody was followed by ELISA. Fractions demonstrating VWF immunoreactivity were pooled and concentrated by precipitation with

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Abbreviations used in this paper: GP, glycoprotein; HBS, Heps-buffered saline; HUVEC, human umbilical vein endothelial cell; TBS, Tris-buffered saline.

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35% (wt/vol) ammonium sulfate followed by centrifugation at 20,000 g for 30 min at 4°C. The precipitate was then resuspended in a small volume of TBS and applied to a 2.5 × 40-cm Sepharose 4B column. 2-ml fractions collected at a flow rate of 40 ml/h were analyzed for vWF as described above. Immunoreactive fractions were pooled and repurified with 35% ammonium sulfate. The precipitate was resuspended in 1-2 ml of TBS, pH 7.4, and dialyzed against TBS overnight at 4°C. The purity of the vWF was > 90% when analyzed by SDS-PAGE.

Cells. Human umbilical vein endothelial cells (HUVEC) were obtained from cords and maintained in culture for one to three passages for these studies as previously described (13–16). Early passage bovine aortic smooth muscle cells were a kind gift of Dr. David Hajjar (Cornell University Medical College, New York). The primary bovine aortic smooth muscle cells were obtained from fresh aortic specimens and were cultured as previously published (17). The human T cell lymphocyte line SK, was a kind gift from Dr. Jeffrey Lawrence (Cornell University Medical College, New York).

Immunohistology. Surgical specimens for these studies were provided by the Department of Pathology, Hunter Holmes McGuire Veterans Administration Hospital. Tissues were fixed in 0.2 M mercuric chloride, 0.13 M sodium acetate, and 3.7% formaldehyde and then imbedded in paraffin before sectioning and staining. After deparaffinization and rehydration, sections were incubated in methanol containing 0.05% hydrogen peroxide to quench endogenous peroxidase activity. Nonspecific antibody binding was inhibited with nonimmune serum of the same species as the secondary antibody. Antibodies were diluted in buffer containing 10 mM sodium phosphate, 0.15 M sodium chloride, and 0.1% bovine serum albumin, pH 7.4 (PBS). Primary antibody staining was performed with monospecific polyclonal rabbit anti-GPIb as well as the monoclonal anti-glycocalcin, 3G6. After washing in PBS the slides were incubated with a biotinylated secondary antibody, washed again, and incubated with avidin and biotinylated horseradish peroxidase. The slides were developed by exposure to the substrate 3-amino-9-ethylcarbazole and counterstained with hematoxylin.

Western blotting. 1% SDS in PBS was used to prepare cell lysates from gel filtered human platelets, cultured human umbilical vein endothelial cells, cultured bovine aortic smooth muscle cells, and SK cells (a T-cell lymphocytic cell line). The cell lysates were electrophoresed in a 7.5% SDS-PAGE, blotted onto nitrocellulose and probed with a monospecific polyclonal rabbit anti-GPIb antibody followed by detection using a peroxidase-linked secondary antibody.

Biosynthesis. Preconfluent T75 flasks of early passage HUVEC and bovine aortic smooth muscle cells were labeled with tritiated leucine at 75 μCi/ml in leucine free growth medium for 20 h and then solubilized. Fab fragments of the monoclonal anti-GPIb antibody-3G6, conjugated to goat antimouse Sepharose beads were used to prepare labeled immunoreactive material. The beads were eluted in 1% SDS and run reduced on 7.5% SDS-PAGE before autoradiography.

Ristocetin agglutination. HUVEC were resuspended in culture flasks by trituration in PBS containing 10 mM EDTA. The cells were fixed in 3% formaldehyde in PBS for 30 min and then washed in PBS containing 0.1 M glycine and resuspended in PBS. Agglutination was followed in standard platelet agglutination wells (18, 19) in a total reaction volume of 150 μl. Suspensions containing 150,000 cells in 50 μl were then added to agglutination wells containing ristocetin alone (1 mg/ml final concentration) or ristocetin plus purified vWF (20 μg/ml final concentration) or ristocetin and vWF in the presence of 20 μg/ml monoclonal anti-GPIb, AP1 (10). At 2 min the wells were photographed. Similar studies with ristocetin were also performed in the presence of normal plasma and plasma from a patient with severe von Willebrand’s disease.

Binding studies. Purified vWF was radiolabeled with 125I by the chloramine-T method (20). Binding experiments were performed according to the method of Kao (21) with minor modifications. The endothelial cells at confluency were washed with Hepes-buffered saline (HBS) once and incubated with 0.01% EDTA and 0.25% BSA in HBS for 15–20 min at 37°C. The cells were harvested by scraping gently with a rubber policeman. The cell suspension was centrifuged for 10 min at 1,000 g to sediment the cells, which were then washed once with HBS. For some experiments, living cells were washed twice more in HBS before the binding studies. Fixed cells were prepared as described above and were used for binding studies after washing three times in HBS.

Binding studies were performed in duplicate at room temperature in 1.5-ml polypropylene tubes (Brinkman). In most assays, the fixed cells (2–4 × 10^5) were incubated with 5.0 μg/ml 125I-vWF, and 0.02% BSA in the presence or absence of 1 mg/ml ristocetin. Incubations were started by the addition of 125I-vWF. After 60 min mixing, 150 μl of each assay mixture was layered on 300 μl of a 1:1 mixture of silicon oil (Dow Corning 550 and 556; Dow Corning Corp., Midland, MI) and spun for 10 min in a microfuge (Beckman Instruments, Inc., Fullerton, CA). After a 50-μl aliquot of supernatant was removed for quantitation of free 125I-vWF, the rest was aspirated and the tips containing pellets were removed and bound radioactivity counted. Ristocetin-dependent binding was calculated by subtracting the binding observed in the absence of ristocetin from that in the presence of ristocetin.

Results
Antibody specificity. The specificity of the monoclonal antibody 3G6 (raised against glycocalcin) and the polyclonal antibody raised to glycocalcin were examined in Western blots against purified glycocalcin and solubilized platelet protein. Results obtained with the monoclonal 3G6 are shown in Fig. 1. Similar specificity was obtained with the polyclonal antibodies (Figs. 3–5). In addition, in platelet aggregation studies, the polyclonal antisera specifically inhibited ristocetin induced platelet aggregation (data not shown). 3G6 did not inhibit ristocetin induced platelet aggregation. In some studies, the monoclonal antibody AP1, which inhibits ristocetin induced platelet aggregation, was used.

Immunohistology. Consecutive sections through human stomach were fixed and sectioned and stained with a monospecific polyclonal anti-GPIb antibody raised to glycocalcin or with nonimmune antibody. Peroxidase positivity indicating the presence of immunoreactive GPIb was present in the smooth muscle as well as endothelial cell layer of the arteriole and the venule (Fig. 2 a, b). The reactivity of the smooth
muscle layer is demonstrated by the broad band of darker staining in Fig. 2 a. Similar sections through human subcutaneous fat were stained with the monoclonal anti-GPIb-3G6. Peroxidase product was present in capillary endothelium but not in surrounding tissue (Fig. 2 c, d).

Western blotting. Western blotting of cell lysates was performed to further establish the cellular origin of the immunoreactive GPIb. In blots performed on unreduced whole cell lysates, probed with a polyclonal antibody raised to unreduted platelet glycolcalicin, both endothelial cells and the smooth muscle cells demonstrated immunoreactive material that co-migrated with unreduted platelet GPIb at 165 kD; the SK cell line was negative (Fig. 3). While blots of reduced platelet lysates revealed a single band of 150-kD, blots of reduced endothelial cell lysates did not demonstrate any immunoreactive material (Fig. 4) suggesting that the monospecific polyclonal antibody raised to glycolcalicin recognized epitopes that were lost upon reduction of endothelial cell GPIb. Results with a second monospecific polyclonal antibody (11) that was raised to reduced and alkylated purified GPIb alpha were consistent with this. Western blots with the second antibody detected immunoreactive material in reduced as well as unreduced endothelial cell lysates (Fig. 5). The apparent molecular weight of unreduced endothelial cell GPIb was 165 kD, and increased to 170 kD upon reduction, suggesting that it is a single chain molecule with intrachain disulfide bond(s). The immunoreactivity that was observed with this antibody, which recognizes colinear amino acid sequences within platelet GPIb-alpha, suggests that the GPIb-like molecule shares a significant degree of sequence homology with platelet GPIb-alpha.

Biosynthesis of radiolabeled GPIb. To examine the synthesis of GPIb by these cells, preconfluent flasks of human umbilical vein endothelial cells and bovine aortic smooth muscle cells were labeled with [3H]leucine and solubilized. Fab fragments of a monoclonal anti-GPIb were used to immunoprecipitate labeled immunoreactive material and autoradiography was performed following separation by SDS-PAGE (Fig. 6). Both human endothelial, and bovine aortic smooth muscle cells synthesized a 150-kD band that corresponds to the alpha subunit of GPIb. Bands at 60 kD and at 30 kD were seen in both cell lines. In addition, a band migrating with an apparent relative molecular mass (M_r) of 210 was observed in endothelial cells. It is possible that this may correspond to a 210-kD GPIb-like molecule previously described in platelets (22). Control immunoprecipitation studies using normal mouse IgG failed to precipitate these specific bands.

Ristocetin agglutination. To examine the function of the GPIb-like molecule present in these cells, the ability of purified vWF and ristocetin to support the agglutination of endothelial cells was examined. In a manner analogous to platelet agglutination studies (23), cultured human umbilical vein endothelial cells were resuspended, fixed in 3% formaldehyde and washed and then incubated in the presence of 1 mg/ml ristocetin alone or with ristocetin plus purified vWF. Agglutination was ristocetin inducible, and vWF dependent, and was largely inhibited by an anti-GPIb monoclonal antibody, AP1, that inhibits ristocetin induced platelet agglutination (Fig. 7). These findings suggest that the GPIb expressed on these cells is functional as a ristocetin-dependent vWF receptor.

Binding studies. To examine more closely the function of the GPIb-like protein in these cells, we measured the binding of purified radiolabeled human vWF to cultured human umbilical vein endothelial cells, bovine aortic smooth muscle cells, and to the T cell line SK.

Binding of 125I-labeled vWF to HUVEC was ristocetin dependent, with a maximum effect at a ristocetin concentration of 1 mg/ml (Fig. 8). Binding to endothelial cells was saturable, and Scatchard analysis yielded a K_d of 7.9 nM ± 2.9 nM (Fig. 9). It is interesting to note that this value agrees well with those previously published for ristocetin dependent vWF binding to the arteriole and the venule. Sections of human subcutaneous fat were stained with (c) the monoclonal 3G6, or with (d) control ascites. Peroxidase product is present in capillary endothelium but not in surrounding tissue.
platelets (21, 24). The binding was specific, as it was inhibitable by excess cold vWF (Fig. 10 a), excess cold glyocalcicin (Fig. 10 b), as well as anti-GPIb antisera (Fig. 10 c). The IC₅₀ of inhibition by cold vWF agrees well with the Scatchard-derived Kᵦ. The higher IC₅₀ of glyocalcicin suggests that its affinity for vWF may be less than that of native GPIb.

Similar studies were performed with bovine aortic smooth muscle cells and with the T cell line SK. Binding to smooth muscle cells was similar to that observed with endothelial cells but binding to the lymphocyte cell line was minimal. Inhibition by anti-GPIb antibody was less effective with the smooth muscle cell line, suggesting some differences in the availability or affinity of the antibody for the GPIb molecule on these cells (data not shown).

**Discussion**

We have identified and isolated a GPIb-like protein from cultured human umbilical vein endothelial cells and from bovine aortic smooth muscle cells. It is of interest that in these cells, the protein appears to be functional, in that it is capable of supporting ristocetin-dependent vWF binding. The Kᵦ observed for vWF binding in these studies agrees well with the Kᵦ previously published for vWF binding to the platelet surface (21, 24). The migration of the isolated GPIb-like molecule reduced and unreduced, in SDS-PAGE suggests that it is a single polypeptide chain with an intrachain disulfide bond or bonds. The GPIb-like molecule is reactive with a polyclonal antiserum that was raised to reduced and alkylated platelet GPIb-alpha and therefore recognizes colinear amino acid domains. Reactivity of the GPIb-like molecule in endothelial cells and smooth muscle cells was also observed with a monoclonal antibody that was raised to the glyocalcicin portion of GPIb-alpha. No evidence for the presence of GPIb-beta was seen in these studies, but it is difficult to exclude the presence of a beta chain entirely.

In platelets, the vWF-GPIb interaction is critical for initial platelet adhesion, and in clinical states of vWF deficiency or platelet GPIb deficiency, platelet function is abnormal (25). The biological role of the GPIb-like molecules in the two cell types we have studied is not yet defined, but it is interesting to speculate that under certain conditions, vWF binding to these vascular cells may initiate platelet adherence and promote thrombosis. Endothelial cells are known to synthesize and secrete vWF in a constitutive manner (26). In addition, vWF is secreted from stored pools of vWF in an activation dependent...
manner (27). In recent studies, vWF expression on the surface of thrombin stimulated cultured endothelial cells has been observed using fluorescence microscopy (28). In vivo, under conditions that lead to stimulation of endothelial cells and release of vWF, endothelial cell GPIb may mediate the expression of endogenous vWF on the cell surface in concentrations that favor platelet adherence. Endothelial cell GPIb might also play a role in thrombosis observed in patients with heparin-associated thrombocytopenia. Recently, antibodies with specificity for HUVEC have been described in patients with heparin-associated thrombocytopenia (29) and heparin-associated platelet aggregation in some patients may be dependent on platelet GPIb (30). It is possible that in some patients with heparin associated thrombocytopenia, antiendothelial cell antibodies are in fact reactive with endothelial cell GPIb.

The presence of a 210-kD band in the endothelial cell immunoprecipitation experiments is of some interest. A 210-kD GPIb-like molecule has been previously described in platelets (22), and may represent a precursor molecule. A patient with antibodies to this protein recently has been described who clinically manifested an acquired Bernard-Soulier syndrome (31). The function of this 210-kD protein in platelets and in endothelial cells remains to be determined. The lower molecular weight species that we observed in immunoprecipitation experiments were likely associated with the immunoprecipitated 150-kD alpha chain, since they were not observed in the Western blots. It is tempting to speculate that in endothelial cells, the 210-kD precursor molecule may be processed to the lower molecular weight GPI proteins: alpha, and beta, with a 60-kD cleavage byproduct. Alternatively, the 60-kD species may be a precursor of fully glycosylated GPIb alpha. A GPIb alpha-like molecule with an apparent Mr of 60,000 has been isolated from HEL cells (32), and recently sequenced (11, 33). In HEL cells the 60 kD molecule appears to correspond to an unglycosylated form of GPIb-alpha.

Recently, the glycoprotein complex IIb/IIIa, which functions on the platelet membrane as a fibrinogen receptor, has been described in several cell types (6–8). Preliminary sequence data supports the premise that IIb/IIIa belongs to a class of adhesive protein receptors that are distributed widely among many cell types (34). We have recently identified a glycoprotein that functions as a receptor for thrombospordin, a platelet alpha granule constituent with a broad tissue distribution. The thrombospordin receptor, which appears to be identical with platelet glycoprotein IV, is present not only in platelets, but also on endothelial cells, monocytes, as well as several tumor cell lines (35). These studies, as well as the observation that GPIb alpha is functional in endothelial cells and in smooth muscle cells, suggests that there are a number of adhesive protein receptors that mediate cell adhesion phenomena and that platelet aggregation may provide a useful paradigm for the understanding of these events. The role of GPIb in mediating adhesion in other cell systems may involve ligands other than vWF. The close association of GPIb with the cytoskeleton makes it a good candidate for a cell receptor in-
involved in the migration of cells. Similar receptor cytoskeleton links are important in other systems (3–5, 36–38). vWF or homologous ligands within the extracellular matrix or on other cells may mediate or modulate cell-substrate attachment and cell-cell interaction in developing organisms or in atherosclerosis, or in tumor cell metastasis.

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Note added in proof. Since the acceptance of this manuscript, the presence of endothelial cell GPIIb has been reported by Sprandio et al. (39).

References


