Synthesis by Cultured Human Umbilical Vein Endothelial Cells of Two Proteins Structurally and Immunologically Related to Platelet Membrane Glycoproteins IIb and IIIa

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Abstract. Human platelets participate in a number of adhesive interactions, including binding to exposed subendothelium after vascular injury, and platelet-platelet cohesion to form large aggregates. Platelet membrane glycoproteins (GP) IIb and IIIa constitute a receptor for fibrinogen that, together with fibrinogen and calcium, is largely responsible for mediating the formation of the primary hemostatic plug. Using highly specific polyclonal and monoclonal antibodies as probes, we could detect the presence of both of these glycoproteins in cultured human umbilical vein endothelial cells. Western-blot analysis showed that the endothelial cell analogues were similar in size to their platelet counterparts, and were present in cells that had been in culture for over 2 mo. Metabolic labeling of endothelium with [35S]methionine demonstrated that both GPIIb and GPIIIa were actively synthesized in culture. Using the technique of crossed immunoelectrophoresis, evidence was obtained that the endothelial cell forms of GPIIb and GPIIIa may exist complexed to one another after solubilization in Triton X-100. The presence of GPIIb-IIIa analogues in cultured endothelial cells may provide an opportunity to examine the structure, function, and synthesis of these two membrane glycoproteins, as well as provide a source of genetic material with which to begin detailed molecular genetic studies.

Materials and Methods

Cells

Human umbilical vein endothelial cells were cultured in complete medium consisting of RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 20% fetal calf serum, 5.9 g/liter Hepes, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin, using previously described methods (11) that are described in detail by McCarroll et al (18). Secondary cultures were prepared by splitting primary cultures 1:3, and were subcultured in the
The Journal of Cell Biology, Volume 103, 1986 82

Electrophoresis and Immunoblotting

All operations were performed at room temperature except where indicated. For Western-blot analysis, cell lysates were diluted 1:1 with a buffer containing 4% SDS, 250 mM Tris, 20% glycerol (pH 6.8), and electrophoresed through a 7% polyacrylamide gel using the buffer system of Laemmli (16). After electrophoresis, proteins were transferred at 4°C to nitrocellulose according to the method of Towbin (34). Sample strips were stained with Amido Black to verify efficient transfer of both large and small molecular weight proteins. The remainder of the nitrocellulose was incubated with blocking buffer (3% gelatin dissolved in 500 mM NaCl and 20 mM Tris, [pH 7.5]) for 30-60 min to block unreacted sites. Nitrocellulose strips were then incubated for 18 h in blocking buffer (20 mM Tris, 500 mM NaCl and 1% gelatin [pH 7.5]) containing either polyclonal or monoclonal antibody at a final concentration of 30 μg/ml. Strips were then washed twice for 10 min each in blotting buffer, incubated with a 1:2,500 dilution of peroxidase-conjugated goat anti-rabbit IgG. (C) Same as in B, but stained with normal rabbit serum. Lanes 1 contain whole solubilized platelet proteins. Lanes 2 contain whole solubilized endothelial cell proteins. Lanes 3 contain fresh endothelial cell growth medium containing 20% horse serum. Color development proceeded via the chromogenic peroxidase substrate, 4-chloro-1-naphthol.

Immunoprecipitation

Endothelial cells were metabolically labeled by first incubating washed confluent cultures with methionine-free RPMI 1640 for 2 h, followed by an additional hour in 10 ml of the same medium containing 250 μCi of [35S]methionine (New England Nuclear, Boston, MA) (specific activity, 1,000 Ci/mmol). After washing twice with RPMI 1640 containing 1 mg/ml cold methionine, the cells were put back into culture for an additional 18 h in the presence of complete medium. Triton lysates were prepared as described above and precleared by a 30-min incubation with normal rabbit IgG, followed by a 5-min incubation with Protein A-Sepharose beads. The beads were removed by centrifugation and indirect immunoprecipitation analysis was performed on the supernatants by incubating ∼500,000 cpm of labeled protein with 2 μl (10 μg) of specific antibody. The same amount of normal rabbit IgG was added to a duplicate tube to serve as a control. After a 2-h incubation, 100 μl of a 50% slurry of Protein A-Sepharose was added, and allowed to bind immune complexes for an additional 30 min. The beads were washed exhaustively in a buffer containing 50 mM Tris, 150 mM NaCl, 1 mg/ml methionine, 1% bovine serum albumin (BSA), and 0.1% SDS (pH 7.2), and bound proteins eluted by boiling the beads in 150 μl of 0.1% SDS, 2% SDS, and 10% glycerol (pH 6.8). Samples were analyzed by SDS-PAGE in 7% polyacrylamide slab gels, followed by fluorography against Kodak XRP-1 x-ray film for 5-30 d at −80°C. Molecular weights were determined by comparison to known standards (BioRad Laboratories).

Crossed Immunoelectrophoresis

CROSSED IMMUNOELECTROPHORESIS (CIE) was performed by mixing 100 μg of Triton X-100-solubilized platelet proteins with 500,000 cpm (∼5 μg protein) of [35S]methionine-labeled endothelial cell lysates and electrophoresing the mixture at 10 V/cm for 75 min at 16°C in 1% agarose dissolved in 38 mM Tris, 100 mM glycerol, and 0.5% Triton X-100 (pH 8.8). Strips of agarose containing the separated proteins were then run at 90° into a 7-cm gel containing rabbit anti-whole platelet antibody for an additional 18 h at 2 V/cm. Immunoprecipitation arcs were visualized by staining with Coomassie Blue R-250. Radiolabeled arcs were identified by exposing dried CIE plates that had been treated with Enhance Spray (New England Nuclear, Boston, MA) to Kodak XRP-1 film as described above, and comparing the resulting autoradiographs to the Coomassie-stained plates.

Results

Identification of Platelet Membrane GPIIb and GPIIIa Analogues in Endothelial Cells

Human umbilical vein endothelial cells were maintained in culture for 3 wk and split 1:3 at the end of each week before

**Figure 1.** Western-blot analysis of nonreduced platelet and endothelial cell proteins. 50 μg of protein was separated on 7% polyacrylamide slab gels and transferred to nitrocellulose. (A) Amido-Black-stained lanes. (B) Immunologically stained with rabbit polyclonal anti-platelet GPIIb-IIIa antibody followed by peroxidase-conjugated goat anti-rabbit IgG. (C) Same as in B, but stained with normal rabbit serum. Lanes 1 contain whole solubilized platelet proteins. Lanes 2 contain whole solubilized endothelial cell proteins. Lanes 3 contain fresh endothelial cell growth medium containing 20% horse serum. Color development proceeded via the chromogenic peroxidase substrate, 4-chloro-1-naphthol.

**Antibodies**

Polyclonal antiserum with specificity for human platelet membrane GPIIb and GPIIIa was prepared in rabbits by twice-monthly intradermal injection of SDS-denatured GPIIb-IIIa that had been purified using a previously described Triton X-114 phase-extraction procedure (22). Purified IgG was obtained by combining ammonium sulfate fractionation and DEAE cellulose chromatography using standard techniques. The specificity of this preparation was confirmed by immunoprecipitation of radioiodinated, solubilized platelets, by crossed immunoelectrophoresis vs. solubilized whole platelets, and by Western-blot analysis. All three methods indicated that most of the antibodies reacted with GPIIb and GPIIIa, with minor reactivity against GPIV also present. For unknown reasons, reactivity with a 200,000-Mr protein was also occasionally observed.

To investigate the presence of GPIIIa in various preparations, a well characterized murine monoclonal antibody, AP3, was used as a specific probe. The properties and purification of this antibody have been previously described in detail (23). AP3 has specificity for GPIIa both complexed to and dissociated from GPIb, reacts equally well with both allelic forms of GPIIa, PI4 and PI5, and binds SDS-solubilized nonreduced GPIIIa that has been immobilized onto nitrocellulose.
preparation of Triton lysates to eliminate any possibility of platelet contamination derived from blood during harvesting from the umbilical vein. Fig. 1 A shows the total lysate protein derived from purified human platelets (lane 1) and twice-passaged human endothelium (lane 2) that has been resolved on a 7% SDS polyacrylamide slab gel, transferred to nitrocellulose, and stained with Amido Black. It is evident from these two lanes that very efficient transfer of both large (>250,000 D) and small (<10,000 D) proteins has occurred. Fig. 1 B represents an identical blot that has been immunologically stained with a polyclonal antibody raised against purified platelet membrane GPIIb and GPIIIa. Previous work of many investigators (6, 15, 19) has established that GPIIb (nonreduced Mr, 136,000) is composed of an α-chain (Mr, 125,000) that is disulfide-linked to a smaller β-subunit (Mr, 21,000). On the other hand, GPIIIa contains numerous intrachain disulfide bonds that, upon reduction, result in an increase in Mr (nonreduced Mr, 90,000; reduced Mr, 105,000). As shown in lane 1, both platelet GPIIb and GPIIIa react strongly with the polyclonal rabbit antibody, as expected. Lane 2 contains an endothelial cell lysate, and shows that two proteins having similar or identical molecular masses to GPIIb and GPIIIa also stain with this antibody. Unlike its platelet counterpart, however, the GPIIIa-like molecule stained less intensively than the GPIIb analogue, and its mobility appeared to be consistently higher than platelet GPIIIa, though only slightly. This differential staining intensity was not always observed, and might be due to spurious proteolysis of the GPIIIa analogue. Greater antigenic cross-reactivity between GPIIb and its endothelial cell analogue would also yield similar results. Two other as yet unidentified endothelial cell proteins were detected by Western blotting, having approximate molecular masses of ~66,000 and 105,000 D. As a control for the possible presence of cross-reactive equine platelet membrane fragments in the horse serum used in culturing the endothelium, Complete Medium (see Materials and Methods) was analyzed for reactivity in lane 3. As shown, only one band at 170,000 D was stained, probably corresponding to horse IgG that cross-reacted with the peroxidase-conjugated anti-rabbit IgG second antibody reagent used in the detection of immune complexes on the nitrocellulose paper.

To confirm and extend these results, platelet and endothelial cell lysates were subjected to further Western-blot analysis using the GPIIIa-specific monoclonal antibody, AP3, as a specific probe. As Fig. 2 shows, AP3 reacted with a single endothelial cell protein that was nearly identical in molecular weight to platelet GPIIIa, further supporting the relationship between these two proteins. Based upon the results shown in Figs. 1 and 2, we adopted the convention suggested by Thiagarajan et al. (33) to term the endothelial cell analogue of platelet GPIIb-IIIa, EC GPIIb, and EC GPIIIa, respectively.

**Figure 2.** Western-blot analysis of nonreduced platelet and endothelial cell lysates. (A) Immunologically stained with polyclonal rabbit anti–platelet GPIIb-IIIa, as in Fig. 1 B. (B) Overlaid with 30 μg/ml of the anti–platelet GPIIb-IIIa-specific monoclonal antibody, AP3, followed by peroxidase-conjugated rabbit anti-mouse IgG. P, lane containing 50 μg whole platelet protein. E, lanes containing 50 μg endothelial cell lysate protein. The decreased mobility of the leftmost portion of the GPIIIa band in lane P is due to partial disulfide-bond reduction resulting from its proximity to mercaptoethanol-containing samples (not shown) that were run adjacent to this lane. This shift in mobility upon reduction is characteristic of GPIIIa (see Fig. 3).

**Figure 3.** Indirect immunoprecipitation of [35S]methionine metabolically labeled endothelial cells. Confluent cell cultures were labeled, lysed with Triton X-100, and precleared by sequential addition of normal rabbit IgG and Protein A-Sepharose. Precleared supernatants were then incubated with rabbit anti–platelet GPIIb-IIIa (lane 1) or normal rabbit IgG (lane 2). Immune complexes were recovered by a 30-min incubation with Protein A-Sepharose, eluted off the beads by boiling in an SDS-containing solution (see Materials and Methods), and analyzed by SDS-PAGE and fluorography. Lane 3 contains ~20,000 cpm of whole radiolabeled endothelial cell lysate. (A) Samples analyzed following treatment with 5% 2-mercaptoethanol. (B) Identical samples as in A, but analyzed nonreduced. Arrows denote the positions, from top to bottom, of EC GPIIb and EC GPIIIa, respectively. Molecular mass markers are shown at right in kilodaltons.

**Metabolic Labeling of EC GPIIb and EC GPIII**

To demonstrate that these two molecules were actively being synthesized by cultured endothelium, cells were cultured in the presence of [35S]methionine, lysates prepared, and indirect immunoprecipitation analysis performed. Fig. 3 shows the results of one such experiment. Fig. 3 A shows preparations analyzed under reducing conditions, while Fig. 3 B shows the same samples analyzed under nonreducing conditions. When total radiolabeled lysate proteins (lane 3) were incubated with rabbit anti–platelet GPIIb-IIIa antibody,
both EC GPIIb and EC GPIIIa were specifically immunoprecipitated (lane 1). Normal rabbit IgG did not react at all with these two proteins (lane 2).

Crossed Immunoelectrophoresis

To explore the possibility that EC GPIIb and EC GPIIIa exist in a complex after solubilization in Triton X-100, and to further explore the structural and immunological relationship between these two proteins and their platelet analogues, unlabeled platelet lysates were mixed with [35S]methionine metabolically labeled endothelial cell lysates in the presence of excess cold methionine, and the mixture analyzed by CIE using a rabbit anti-whole platelet antibody preparation in the second dimension. As Fig. 4A shows, Coomassie Blue staining revealed the presence of a major precipitin arc that has been shown to contain the calcium-dependent GPIIb-IIIa complex (14). It has been previously shown that chelation of calcium results in dissociation of the complex, disappearance of the GPIIb-IIIa arc, and the concomitant appearance of two new precipitin arcs containing the individual glycoproteins (8, 14). Thus, the appearance in the corresponding autoradiograph (Fig. 4B) of a single precipitin arc that is virtually superimposable with the platelet GPIIb-IIIa complex strongly indicates that EC GPIIb and EC GPIIIa exist complexed in Triton lysates of human endothelium. Furthermore, when endothelial cell lysates were analyzed by CIE without prior admixture with solubilized platelets (not shown) the precipitin arc formed by the GPIIb-IIIa complex was still prominent, though it represented only 5-10% of the area given by the corresponding arc shown in Fig. 4, reflecting the proportionately decreased amount of this glycoprotein complex in endothelial cells relative to that found in platelets.

Discussion

Human endothelial cells have previously been shown to synthesize several proteins that are also present in circulating blood platelets, including von Willebrand factor (9, 36), von Willebrand Antigen II (18), thrombospondin (21, 28), and fibronectin (10). In this paper, we have shown that endothelial cells also synthesize a protein that is both structurally and immunologically related to platelet membrane GPIIb, and have confirmed an earlier report (33) that they synthesize its membrane counterpart, GPIIIa. Moreover, we have provided evidence that these two membrane glycoproteins exist complexed to each other after solubilization in Triton X-100, as is the case for GPIIb-IIIa in human platelets.

Using a polyclonal antibody with specificity for denatured GPIIb and GPIIIa allowed us to detect the presence of analogues of both of these two platelet membrane glycoproteins. In contrast, Thiagarajan et al. (33) used both a monoclonal and a polyclonal antibody, each having specificity for only GPIIIa, to detect a related protein in endothelial cells. Though these authors suggested that a GPIIb-like molecule was unlikely to be present in cultured endothelium, their argument was in part based upon their unpublished observations that the anti-platelet GPIIb-specific monoclonal antibody, Tab (19, 20), was unreactive with endothelial cells. It is possible, however, that the epitope on GPIIb defined by Tab has not been conserved in its endothelial cell analogue. Since GPIIb is a large glycoprotein (mol mass, 136,000), it is likely that several regions on the molecule could be subtly modified without significantly altering its overall structure and function.

Support for the presence of a complex between EC GPIIb and EC GPIIIa was generated by our finding a metabolically labeled precipitin arc derived from endothelium that mi-

Figure 4. CIE of a mixture of platelet and endothelial cell proteins. 100 mg of unlabeled platelet lysate protein was mixed with 500,000 cpm (~5 μg) of [35S]methionine-labeled endothelial cell protein, and the mixture separated by CIE into an agarose gel containing polyclonal rabbit anti-whole platelet antibody. (A) The resulting CIE plate stained with Coomassie Blue. (B) The fluorograph derived by exposure of the CIE plate. The arrow denotes the major precipitin arc formed by the GPIIb-IIIa complex. Note that several Coomassie-stained arcs are not radiolabeled, and vice versa.
igrated identically with the platelet GPIIb-IIIa complex in CIE gels (Fig. 4). By mixing together the two samples, one labeled and the other not, before their two-dimensional separation based upon first size and charge, and then upon immunologic cross-reactivity, we were able to demonstrate homology between the precipitin arcs given by the platelet and endothelial cell IIB and IIIA. Further studies are necessary to determine the precise structural and physical requirements for IIB-IIIa complex formation in these two cell types.

GPIIb/IIIa-like molecules have now been identified in several varied cell types (7, 15, 32, 33). Moreover, while this paper was in review, a Communication appeared (5) indicating that human endothelial cells synthesize two glycoproteins similar to platelet GPIIb and GPIIIa, thereby independently confirming the results presented in this paper. Since the primary role of the GPIIb-IIIIa complex in platelets is mediating platelet aggregation through its fibrinogen-receptor activity, it is reasonable to wonder what the possible role of an analogous receptor might be in human transformed cell lines (7, 32), or on the supposedly hemostatically inert endothelial cell surface (5, 33, this paper). While we do not yet know, we might speculate that this large heterodimer complex could support several varied functions, of which only several might play a role in any given cell type. Brass and Shattil (4), and Brass (3) have shown that GPIIb and GPIIIa represent the major Ca++-binding surface glycoproteins in resting platelets, and may play a role in Ca++ transport. Perhaps they have similar functions in other cell types. Shade et al. (31) recently demonstrated that GPIIb plays a role in the adherence of platelets to collagen. It is, therefore, possible that its analogue might play an important role in mediating the normal attachment of endothelial cells to the collagen-rich subendothelial matrix of the vascular wall.

Preliminary results obtained in our laboratory indicate that EC GPIIIa also bears the human platelet alloantigen, P1AI (12) (to be published in detail elsewhere). Since the P1AI antigenic determinant has recently been localized to a 17-kD region of GPIIIa (24), these results provide further support for the homology shared by GPIIIa and EC GPIIIa. Furthermore, this finding might have important implications regarding the etiology of such alloimmunological disorders as posttransfusion purpura, neonatal alloimmune thrombocytopenia, and autoimmune thrombocytopenia, where antibodies directed against either GPIIb or GPIIIa, or both, mediate the immune destruction of circulating platelets. Studies are in progress to further investigate this question.

We have found that EC GPIIb and EC GPIIIa continue to be synthesized by cultured endothelium for at least eight passages (2 mo) (data not shown). Long-term maintenance of endothelial cells in culture allows large numbers of these cells to be grown and harvested, raising the possibility of preparing cDNA libraries that would contain genes coding for EC GPIIb and EC GPIIIa. Eventual cloning of these specific genes should allow for their comparison with similar proteins found in platelets and other cells, and could yield important clues regarding the structural requirements for their function(s) in these varied cell types.

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