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A CD44-Like Endothelial Cell Transmembrane Glycoprotein (GP116) Interacts with Extracellular Matrix and Ankyrin

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We used complementary biochemical and immunological techniques to establish that an endothelial cell transmembrane glycoprotein, GP116, is a CD44-like molecule and binds directly both to extracellular matrix components (e.g., hyaluronic acid) and to ankyrin. The specific characteristics of GP116 are as follows: (i) GP116 can be surface labeled with $Na^{125}I$ and contains a wheat germ agglutinin-binding site(s), indicating that it has an extracellular domain; (ii) GP116 displays immunological cross-reactivity with a panel of CD44 antibodies, shares some peptide similarity with CD44, and has a similar 52-kDa precursor molecule, indicating that it is a CD44-like molecule; (iii) GP116 displays specific hyaluronic acid-binding properties, indicating that it is a hyaluronic acid receptor; (iv) GP116 can be phosphorylated by endogenous protein kinase C activated by 12-*O*-tetradecanoylphorbol-13-acetate and by exogenously added protein kinase C; and (v) GP116 and a 20-kDa tryptic polypeptide fragment of GP116 from the intracellular domain are capable of binding the membrane-cytoskeleton linker molecule, ankyrin. Furthermore, phosphorylation of GP116 by protein kinase C significantly enhances GP116 binding to ankyrin. Together, these findings strongly suggest that phosphorylation of the transmembrane glycoprotein GP116 (a CD44-like molecule) by protein kinase C is required for effective GP116-ankyrin interaction during endothelial cell adhesion events.

Endothelial cells are known to function in a variety of important physiological processes. In particular, they are responsible for the regulation of blood vessel permeability and tone as well as being involved in blood coagulation. Essentially all of the endothelial cell functions involve interactions with neighboring cells and/or extracellular matrix, in which the endothelial cell surface components must play an important role (4, 15, 18). In vitro studies indicate that glycoprotein GPIIb-IIIa, a member of a family of adhesion receptors called integrins, exists on the surface of endothelial cells (33). Integrins are transmembrane glycoproteins known to bind both extracellular matrix components, such as fibronectin (10, 21), laminin (10), and vitronectin (10), and cytoskeletal proteins, such as talin and vinculin (23), in adhesion plaques. Although plaque structures have not been observed in endothelium in vivo, the cytoplasmic aspect of endothelial cell junctions does include a dense peripheral band of actin filaments which is associated with vinculin (40). These studies suggest that transmembrane interactions probably occur between adhesive receptor molecules and the underlying cytoskeleton in endothelial cells.

Recent evidence indicates that phosphorylation of certain cytoskeletal proteins may be involved in regulating the transmembrane interactions occurring between adhesive receptors and cytoskeletal components. For example, phosphorylation of vinculin (36), talin (14, 34), and integrin (22) at tyrosine residues in certain virally transformed (nonendothelial) cells appears to cause the disassembly of the adhesive receptor-cytoskeleton complex and destabilization of the adhesion plaque. However, the validity of such a conclusion is still in question, since there are mutant virus strains which do not alter the adhesion plaque formation or cell morphology following tyrosine phosphorylation of vinculin (27) and talin (14, 34). Nevertheless, protein kinase C (PKC) activity has been recently found to play an important role in regulating the interaction between the lymphoma homing receptor GP85 (which is identical to CD44 and Pgp-1) and ankyrin (26). With regard to endothelial cells, relatively little is known concerning the possible interactions which may occur between surface adhesive molecules and cytoskeletal proteins. In this regard, we have now identified a new endothelial cell surface adhesion molecule, GP116, which displays the following properties: (i) it has antigenic determinants which are either very similar or identical to those of CD44, as defined by a panel of anti-CD44 antibodies; (ii) it contains specific hyaluronic acid (HA)-binding sites; (iii) it has transmembrane glycoprotein properties; (iv) it can be phosphorylated in its cytoplasmic domain by PKC; and (v) it has an ankyrin-binding site in its cytoplasmic domain. Most importantly, phosphorylation of GP116 by either endogenous PKC (activated by 12-*O*-tetradecanoylphorbol-13-acetate [TPA]) or exogenous PKC significantly enhances the binding of GP116 to the membrane linker molecule, ankyrin.

MATERIALS AND METHODS

Cell culture. The endothelial cells used in this study are from a chemically transformed bovine aorta cell line (GM 7372A) which was obtained from the Institute for Medical Research, Camden, N.J. The cells were cultured in Eagle minimal essential medium with Earle's salts, twofold-concentrated essential and nonessential amino acids, twofold-concentrated vitamins, plus 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. Confluent cultures were passaged by using trypsin-EDTA to detach the cells and then plated at a 1:8 dilution onto uncoated, plastic tissue culture surface. The endothelial cells grew with a population doubling time of

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about 30 h and formed a flat, cobblestonelike monolayer (20).

Preparation of [^{3}H]HA. A rat fibrosarcoma cell line (kindly provided by Charles Underhill, Georgetown University, Washington, D.C.) was grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum, penicillin (1%), and streptomycin (1%) at 37°C in 5% CO_2–95% air. When cells reached to 70 to 80% confluence in 100-mm-diameter dishes, the growth medium was replaced with DMEM containing 2% fetal bovine serum, and the cells were incubated with D-[^{3}H]glucosamine (10 μCi/ml; specific activity, 10 to 20 Ci/mol) for 72 h. The culture medium was collected, centrifuged, and dialyzed extensively against distilled water. [^{3}H]HA was prepared as described previously (38). The specific activity of [^{3}H]HA was estimated to be 80 × 10^3 to 100 × 10^3 dpm/μg of HA. The concentration of [^{3}H]HA was determined by a modified uronic acid carbazole reaction (5).

Radioactive labeling of cellular proteins. Both surface proteins on intact cells and isolated cellular proteins (e.g., GP116 and GP85 [CD44]) were labeled with Na^{125}I, using the Iodo-Gen method of Fraker and Speck (17).

Plasma membrane isolation. Surface-^{125}I-labeled or unlabeled endothelial cells were scraped from tissue culture flasks and washed three times with phosphate-buffered saline (PBS) (pH 7.3). Endothelial plasma membranes were isolated by the fractionation scheme outlined by Bourguignon et al. (7–9).

Phorbol ester treatments and protein phosphorylation. Phorbol esters and their derivatives, such as TPA and 4-α-phorbol-12,13-didecanoate (4α-PDD; Sigma), were used to examine the phosphorylation of membrane proteins, which were obtained by the following two methods.

(i) In vivo phosphorylation. To metabolically label cellular phosphoproteins, endothelial cells were washed with phosphate-free DMEM and then incubated with 0.25 mCi of $H_3{}^{32}PO_4$ (carrier free; ICN Radiochemicals) per ml for 1 h at 37°C (isotopic equilibrium inside the cell is reached under this condition). In some cases, cells were phosphorylated in vivo under identical conditions except using non-radioactively labeled H_3PO_4. Radioactively labeled or unlabeled cells were subsequently exposed to TPA or 4α-PDD (10^{-7} M) or phosphate-free DMEM alone for 10 min at room temperature. Plasma membrane fractions were isolated and used for the purification of GP116 or analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and autoradiography as described below.

(ii) In vitro phosphorylation. Plasma membranes were incubated in a solution containing 20 mM Tris-HCl (pH 7.4), 2 mM dithiothreitol, 10 mM magnesium acetate, 0.5 mM $CaCl_2$, and 1 mM [τ-^{32}P]ATP (100 to 200 cpm/pmol) in the presence of either TPA or 4α-PDD (10^{-7} M) for 10 min at room temperature. In some cases, plasma membranes were phosphorylated in vitro under identical conditions except using non-radioactively labeled ATP. Since all of the phorbol ester-treated samples contain 1% dimethyl sulfoxide, 1% dimethyl sulfoxide (in PBS) was also added to all control samples. Radioactively labeled and unlabeled plasma membranes were used for the purification of GP116 or analyzed by SDS-PAGE and autoradiography as described below.

Stoichiometry analysis. GP116 (50 μg) purified as described below was incubated in a solution containing 20 mM Tris-HCl (pH 7.4), 2 mM dithiothreitol, 10 mM magnesium acetate, 0.5 mM $CaCl_2$, and 1 mM [τ-^{32}P]ATP (100 to 200 cpm/pmol) plus membrane-associated endogenous endothelial cell PKC (in the presence or absence of 4α-PDD/TPA) or brain PKC. Subsequently, phosphorylated GP116 was separated from endothelial membrane by 100,000 × g_{av} centrifugation and then subjected to anti-CD44-conjugated immunoaffinity column chromatographic and SDS-PAGE analyses as described below. The band corresponding to phosphorylated GP116 was cut out, and its phosphate content (expressed in moles) was determined on the basis of the specific activity of [γ-^{32}P]ATP (100 to 200 cpm/pmol) used in this assay. The amount of protein in the phosphorylated GP116 band was determined by scanning densitometry of a silver-stained gel calibrated with protein standards. This value was converted into moles of GP116 and compared with the moles of PO_4 present in the GP116 band.

Triton X-100 extraction of endothelial cell membranes. Purified plasma membranes (non-radioactively labeled, surface ^{125}I labeled, or ^{32}P labeled) were resuspended in PBS (pH 7.3) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 5 mM iodoacetamide, and 1% Triton X-100. Samples were incubated at 4°C for 1 h with constant mixing and then centrifuged at 43,000 × g_{av} for 20 min. The pellet (Triton X-100-insoluble material) was discarded, and the supernatant (Triton X-100-soluble material) was collected for use in wheat germ agglutinin (WGA) column chromatography.

WGA-Sepharose column chromatography. Triton X-100-solubilized plasma membranes (non-radioactively labeled, surface ^{125}I labeled, or ^{32}P labeled) were mixed with WGA-Sepharose beads overnight at 4°C with constant mixing. The beads were then packed into a column and washed with 20 volumes of PBS (pH 7.3) to remove unbound material. The WGA-binding proteins were then eluted from the column with a PBS solution containing 0.75 M *N*-acetylglucosamine and 0.1% Triton X-100. Control experiments indicated that other unrelated saccharides, such as *N*-acetylgalactosamine (0.75 M), failed to elute WGA-binding proteins. The WGA-binding proteins were then used for the adhesion protein binding experiments, for the immunoaffinity column chromatography experiments, and for SDS-PAGE and autoradiographic analyses.

Purification of GP116 by immunoaffinity column chromatography. WGA-binding proteins (non-radioactively labeled, surface ^{125}I labeled, or ^{32}P labeled) were added to monoclonal rat anti-human CD44-Sepharose beads (CMB-Tech, Inc., Miami, Fla.) and allowed to bind overnight. The beads were then transferred to a column and washed extensively with PBS (pH 7.4) containing 0.05% Triton X-100. The bound material (purified GP116) was eluted with a solution containing 0.05 M diethylamine, 10 mM EDTA, and 0.05% Triton X-100 (pH 11.5). A portion of the eluted materials (purified GP116) was analyzed by SDS-PAGE and autoradiography as described below.

Immunoprecipitation. Surface-^{125}I-labeled endothelial cells were solubilized in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS) containing 5 mM iodoacetamide, 1 mM PMSF, and 10 μg of leupeptin per ml. The solubilized cell extracts were incubated with one of the following immunoreagents: (i) IM7 (rat anti-mouse CD44) (30), (ii) IRAWB 14 (rat anti-mouse CD44) (29), (iii) Leu 44 (mouse anti-human CD44) (Becton Dickinson, San Jose, Calif.), or (iv) normal rat or mouse immunoglobulin G (IgG) (as a control) at 4°C for 15 h. The extracts were then incubated with goat anti-rat IgG-agarose beads (for IM7, IRAWB 14, and control samples) or goat anti-mouse IgG (for Leu 44) at 4°C for 2 h. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography as described below.

Digestion of GP116 by *N*-glycosidase F. ^{125}I-labeled GP116 (purified from the surface-^{125}I-labeled plasma membranes as described above) bound to the immunoaffinity column was subsequently solubilized in a solution containing 1% SDS and 50 mM sodium phosphate buffer (pH 6.5) and then boiled for 2 min. The SDS-solubilized material was incubated with a solution containing 50 mM sodium phosphate buffer (pH 6.5), 10 mM EDTA, 1% Triton X-100, and 1 U of *N*-glycosidase F (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) at a final SDS concentration of 0.075%. After incubating for 16 h at 37°C, the digested sample was analyzed by SDS-PAGE and autoradiography.

Binding of [^{3}H]HA to endothelial cells (in vivo assay). Endothelial cells ($\approx 5 \times 10^4$) grown in 24-well culture plates were incubated with 1 μg of [^{3}H]HA in the presence of various concentrations of unlabeled HA in binding buffer (PBS [pH 7.4] containing 0.2% bovine serum albumin [BSA]). The binding reaction was carried out at 4° for 2 h. Cell were then washed three times with binding buffer and solubilized with 1% SDS in PBS. To determine cell-associated [^{3}H]HA, SDS-solubilized samples were subjected to liquid scintillation counting. All binding studies were carried out in duplicate.

Extracellular matrix binding assays (in vitro assay). ^{125}I-labeled GP116 (isolated from the surface-iodinated plasma membrane fraction and then passed through the immunoaffinity column) was incubated at room temperature for 30 min with a nitrocellulose sheet coated with either HA or chondroitin sulfate. The extracellular matrix protein-coated nitrocellulose sheets were prepared by soaking the nitrocellulose sheets in a 3-mg/ml solution of each reagent (e.g., HA and chondroitin sulfate) and then air drying them. Following incubation with ^{125}I-GP116, the nitrocellulose sheets were extensively washed with PBS (pH 7.4) and counted in a gamma counter. A 100-fold excess of soluble HA blocks GP116 binding to HA-coated nitrocellulose sheets. Background or nonspecific binding was determined by including a large excess amount of unlabeled extracellular matrix molecule (100-fold excess) in the assay. The results are expressed as specific binding, i.e., binding from which the background level has been subtracted.

Phosphoamino acid analysis. GP116 was phosphorylated by plasma membrane-associated PKC (in the presence of TPA) or brain PKC as described previously (26). Phosphorylated GP116 was then hydrolyzed at 110°C for 2 h by the method of Cooper et al. (12). Phosphoamino acids were separated by one-dimensional thin-layer chromatography on a Kodak chromagram 13255 cellulose sheet at pH 3.5 for 30 min at 1,500 V. The phosphoamino acid standards (phosphoserine, phosphotyrosine, and phosphothreonine) were mixed with the ^{32}P-labeled samples, coanalyzed, and identified by ninhydrin staining. The ^{32}P-labeled phosphoamino acids were detected by autoradiography (X-Omat XAR-5 film at −70°C). As a positive control, we also carried out a phosphoamino acid analysis of the IM-9 B-lymphoblast insulin receptor, showing the presence of phosphoserine, phosphothreonine, and phosphotyrosine.

Ankyrin purification. Human erythrocyte ankyrin was purified by the procedure of Bennett and Stenbuck (2).

Binding of GP116 to Ankyrin-conjugated Sepharose beads. Ankyrin was purified from 40 ml of erythrocyte ghosts by the procedure of Bennett and Stenbuck (2). This protein was then conjugated to cyanogen bromide-activated Sepharose beads as described previously (25, 26). For the ankyrin binding assays, Triton X-100-solubilized plasma membrane or purified GP116 (^{32}P or ^{125}I labeled) was used. ^{32}P-labeled GP116 was obtained from both in vivo and in vitro phosphorylation as described above. In some cases, the unlabeled GP116 was phosphorylated either in vivo or in vitro, using non-radioactively labeled H_3PO_4 or ATP, respectively, as described above. Phosphorylated GP116 was subsequently labeled with ^{125}I by incubation with Na^{125}I, using the Iodo-Gen method (17). These ^{32}P- and ^{125}I-labeled plasma membrane proteins or purified GP116 (2×10^6 cpm per sample) was subsequently incubated with an aliquot of ankyrin-conjugated Sepharose beads (Ankyrin-beads; 100 μl) in a binding solution containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Triton-X 100, and 0.1% BSA at 4°C overnight. As controls, samples were preincubated with soluble ankyrin (50 to 100 μg/ml), which completely blocks binding of plasma membrane proteins or GP116 to ankyrin-beads. After binding, the beads were washed extensively with the binding solution. The results are expressed as amount of specific binding, i.e., binding from which the background level has been subtracted.

Trypsin-treated GP116 was prepared as follows. ^{32}P-labeled GP116 (phosphorylated by either endogenous endothelial cell PKC in the presence of TPA or brain PKC as described above) was incubated with trypsin that was conjugated to Sepharose beads (trypsin-Sepharose or immobilized trypsin) (100 μg/ml) at 35°C for 10 min with constant shaking. The trypsinization reaction was terminated by adding 100 mM PMSF (final PMSF concentration of 10 mM). Subsequently, trypsin-Sepharose beads were centrifuged at $10{,}000 \times g_{av}$ for 4 min. The ^{32}P-labeled trypsin-digested GP116 polypeptide fragments (2×10^6 cpm per sample) were used to bind ankyrin-beads as described above. As controls, samples were preincubated with soluble ankyrin (50 to 100 μg/ml), which completely blocks binding of the 20-kDa proteolytic fragment to ankyrin-beads. After binding, the beads were washed extensively with the binding solution. Background or nonspecific binding was determined by including a large excess amount of soluble ankyrin (50 to 100 μg/ml) in the assay.

One-dimensional peptide mapping. First, purified GP116 or lymphoma GP85 (CD44) (prepared as described previously) (31) was labeled with Na^{125}I, using the Iodo-Gen method (17). Subsequently, various amounts (0, 1, or 5 μg) of staphylococcal V8 protease were added to ^{125}I-labeled GP116 or GP85 (CD44) for 10 min at 30°C to allow proteolytic digestion to occur. Both peptide fragments of endothelial cell GP116 and lymphoma CD44 (GP85) were subjected to SDS-PAGE (10% gel) and autoradiographic analyses as described below.

SDS-PAGE and autoradiography. One-dimensional PAGE was done by using a linear polyacrylamide gradient 5 to 15%, 10%, or 12% slab gel and a discontinuous buffer system as described by Laemmli (28). For autoradiographic analysis, gels were vacuum dried and exposed to Kodak X-ray (X-Omat XAR-5) film.

Analysis of GP116 as a transmembrane protein. Endothelial cells grown in 100-mm-diameter culture dishes to confluence were incubated in L-methionine-free DMEM (GIBCO) at 37°C for 4 h. Cells were then labeled with 200 μCi of Trans-^{35}S label (>1,000 Ci/mmol; ICN) per ml for 5 min. Following labeling, cells were washed three times in PBS, gently scraped from the dishes, and homogenized with a Dounce homogenizer on ice. The nuclei and unbroken cells were removed by centrifugation at $500 \times g_{av}$ for 10 min. The supernatant was centrifuged at $15{,}000 \times g_{av}$ for 20 min to pellet the crude microsomal fraction. This material was further purified through a discontinuous sucrose gradient

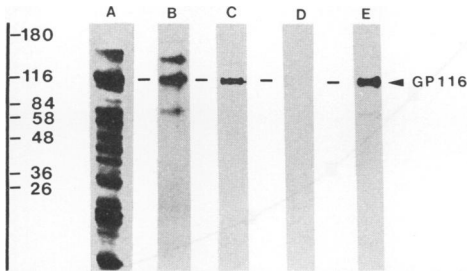

FIG. 1. Analysis of endothelial plasma membrane proteins. Lanes: A, ^{125}I-labeled total plasma membrane proteins; B, ^{125}I-labeled endothelial plasma membrane proteins which were bound to a WGA-Sepharose column and eluted with 0.75 M *N*-acetylglucosamine; C, ^{125}I-labeled GP116 isolated from a rat anti-CD44-conjugated immunoaffinity column; D, nonspecific binding material (labeled with ^{125}I), using a control serum (anti-CD44 free serum) conjugated to the immunoaffinity column; E, ^{125}I-labeled endothelial plasma membrane proteins which were solubilized by Triton X-100 and bound to ankyrin-beads. Sizes are indicated in kilodaltons.

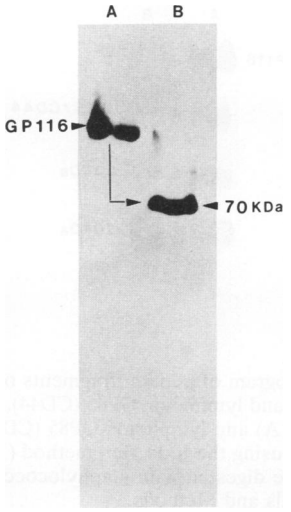

FIG. 2. Digestion of GP116 by *N*-glycosidase F. Surface-^{125}I-labeled GP116 was prepared by rat anti-CD44-conjugated immunoaffinity column chromatography as described in Materials and Methods. Lanes: A, intact GP116 without treatment; B, GP116 digested with *N*-glycosidase F.

(consisting of 20, 30, 40, 50, and 65% [wt/wt] sucrose) and then centrifuged at 100,000 × g_{av} for 2 h. The purified microsomes were collected from the 50-65% sucrose interface and verified by enzyme markers such as NADPH-dependent cytochrome *c* reductase and sulfatase C. The microsome fraction was then washed and resuspended in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2) in the presence or absence of bovine trypsin (100 μg/ml) for 10 min at 37°C. Reaction was terminated by adding SDS (final concentration of 1%) in 20 mM Tris-HCl (pH 7.4)–150 mM NaCl. The solubilized microsomal extracts were boiled for 2 min and diluted in a solution containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% BSA, leupeptin (10 μg/ml), aprotinin (10 μg/ml), and PMSF (1 mM). The solubilized extracts were immunoprecipitated with rat anti-CD44 antibodies as described above. The immunoprecipitates were analyzed by SDS-PAGE and fluorography.

RESULTS AND DISCUSSION

GP116 is a major iodinated plasma membrane glycoprotein. Initially, we used a cell surface ^{125}I-labeling technique to examine the endothelial plasma membrane proteins. There was a major iodinated protein of ≈116 kDa among the large number of labeled proteins present on the endothelial cell surface (Fig. 1, lane A). After solubilizing the ^{125}I-labeled plasma membranes with Triton X-100, we determined that the 116-kDa protein is a glycoprotein, since it binds specifically to a WGA column (lane B). We now designate this protein GP116. We have further characterized this glycoprotein by using *N*-glycosidase F to cleave both high-mannose- and complex-type N-linked oligosaccharides. Our results indicate that GP116 is converted to a 70-kDa peptide after *N*-glycosidase F digestion (Fig. 2). This finding suggests that GP116 is a glycoprotein containing N-linked oligosaccharides (N glycosylation) which represents a relatively large fraction of total molecular weight of this protein.

Cross-reactivity of GP116 with various anti-CD44 antibodies and peptide mapping analysis. The interaction between endothelial cells and lymphocytes in high endothelial venules is an important heterotypic cell-cell interaction required for the recirculation of lymphocytes (3). Recent work from a number of different laboratories has shown that monoclonal antibodies raised against lymphoid antigen CD44 (e.g., IM7) (35), Pgp-1 (32), and the Hermes class of lymphocyte homing receptors (gp90^{Hermes}) (24) all bind to the same antigen. Although this molecule is detected on a wide variety of cells, including lymphocytes, macrophages, epithelial cells, and some neurons (11), it was not certain whether CD44-like antigens are also present on the surface of endothelial cells.

Using a panel of monoclonal antibodies that recognize different epitopes on CD44 (e.g., IM7, IRAWB 14, and Leu 44), we have established that surface-iodinated GP116 displays immunological cross-reactivity with CD44 (Fig. 1, lane C; Fig. 3, lanes A to C). No GP116-associated material is

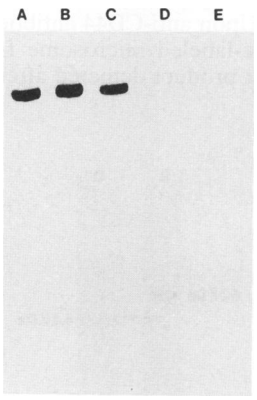

FIG. 3. Autoradiogram of various anti-CD44 antibody-mediated immunoprecipitations of GP116. Surface-^{125}I-labeled endothelial cells were solubilized and immunoprecipitated with rat anti-mouse CD44 (IM7) (lane A), rat anti-mouse CD44 (IRAWB 14) (lane B), mouse anti-human CD44 (Leu 44) (lane C), normal rat IgG (as a control) (lane D), and normal mouse IgG (as a control) (lane E) at 4°C for 15 h and then with goat anti-rat IgG-agarose beads (for IM7, IRAWB 14, and control samples) or goat anti-mouse IgG (for Leu 44) as described in Materials and Methods.

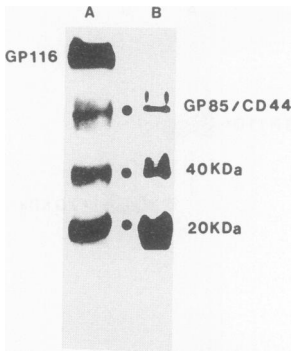

FIG. 4. Autoradiogram of peptide fragments of ^{125}I-Labeled endothelial cell GP116 and lymphoma GP85 (CD44). Purified endothelial cell GP116 (lane A) and lymphoma GP85 (CD44) (lane B) were labeled with Na^{125}I, using the Iodo-Gen method (17). Radioactively labeled samples were digested with staphylococcal V8 protease as described in Materials and Methods.

observed in control samples when either the preabsorbed serum (anti-CD44 free serum) or normal rat or mouse IgG is used (Fig. 1, lane D; Fig. 3, lanes D and E).

Furthermore, results of peptide mapping, using limited staphylococcal V8 protease digestion of GP116 (Fig. 4, lane A), indicate that this protein also shares some peptide similarity with lymphoma GP85 (CD44) (Fig. 4, lane B). These findings are consistent with recent work reported by Favaloro et al. suggesting that there is expression of a CD44-like molecule on vascular endothelial cells (16).

Further analyses using *N*- and *O*-glycosidases indicate that there are detectable differences in the glycosylation patterns between GP116 and bona fide CD44 (data not shown). The possible differences in glycosylation between GP116 and bona fide CD44 may be necessary for various adhesion functions in different cell types. This notion awaits further investigation.

Transmembrane nature of GP116. To fully establish a transmembrane topology for GP116, we also used an in vitro proteolytic treatment of the microsomal fraction obtained from cells metabolically pulse-labeled with [^{35}S]methionine for 5 min (Fig. 5). Upon anti-CD44 antibody precipitation of the [^{35}S]methionine-labeled microsome fraction, a 52-kDa molecule is the first product detected after 5 min of radiolabeling (Fig. 5, lane A). Further pulse-chase experiments indicate that this 52-kDa molecule is the initial biosynthetic precursor of GP116 (data to be published elsewhere). The glycosylation of the 52-kDa protein has not been fully characterized. Nevertheless, a similar 52-kDa polypeptide, which is also a precursor of the CD44 (GP85) molecule, has been reported in mouse T-lymphoma cells (31).

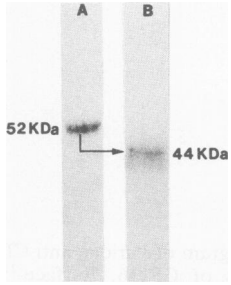

FIG. 5. In vitro proteolytic treatment of the microsomal fraction. Cells were metabolically pulse-labeled with [^{35}S]methionine for 5 min, and the microsomal fraction was isolated. [^{35}S]methionine-labeled microsome fraction in the absence (lane A) or presence (lane B) of trypsin treatment was subsequently solubilized and immunoprecipitated by rat anti-CD44 antibody as described in Materials and Methods.

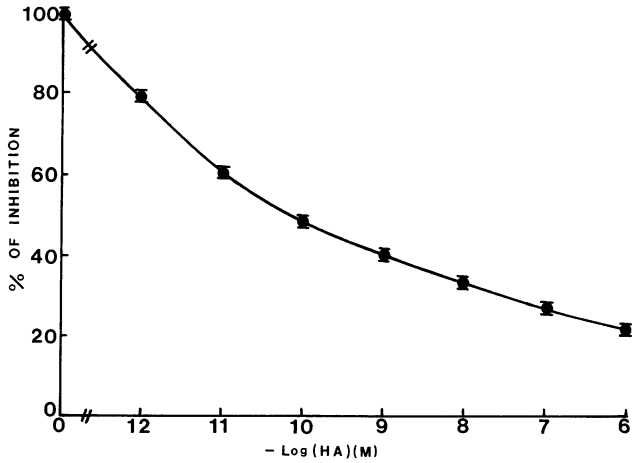

FIG. 6. Binding of [^{3}H]HA to endothelial cells. Endothelial cells ($\approx 5 \times 10^4$) grown in 24-well culture plates were incubated with 1 μg of [^{3}H]HA in the presence of various concentrations of unlabeled HA in binding buffer as described in Materials and Methods.

Extensive treatment of the radiolabeled microsome fraction with trypsin results in one major polypeptide of 44 kDa (Fig. 5, lane B). Therefore, the trypsin-sensitive region of the 52-kDa protein (molecular mass of ≈8 kDa, or 72 amino acids) must represent the cytoplasmic domain of GP116, which is consistent with the deduced nucleotide sequence data for CD44 (35, 39). The facts that (i) several rat anti-CD44 antibodies used in this study have been shown to interact with surface HA-binding sites (29, 30), (ii) the anti-CD44-immunoprecipitable intraluminal domain (e.g., 44 kDa) of GP116 precursor is protected from trypsin treatment (Fig. 5), and (iii) GP116 is glycosylated (Fig. 2) suggest that GP116 is a transmembrane glycoprotein.

Binding of GP116 to extracellular matrix components. Because of its widespread occurrence and its role in cell adhesion, CD44/Pgp-1/gp90^{Hermes} has also been designated a homing cellular adhesion molecule. Although the known homing cellular adhesion molecules have no significant homologies with immunoglobulins or integrins (19), their deduced amino acid sequences indicate a significant homology with the functional domains of the cartilage proteoglycan core and link proteins. These proteins are primarily involved in binding HA (13, 29, 30).

In this study, we have established an in vivo assay using [^{3}H]HA to bind to endothelial cells in the presence of various concentrations of unlabeled HA in order to establish the specificity and affinity of binding to extracellular matrix components such as HA. Our data reveal the presence of a single high-affinity class of receptor with a dissociation constant of 0.1 nM (Fig. 6) which is the same as that reported earlier (38). In an in vitro assay, we also determined that purified GP116 binds HA (repeating disaccharide of D-glucuronic acid and *N*-acetyl-D-glucosamine) (specific binding of 1,900 cpm [average of three experiments with standard deviation of $< \pm 5\%$]) but not chondroitin sulfate (repeating

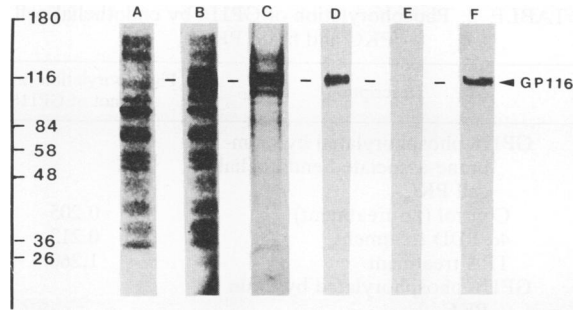

FIG. 7. Effects of phorbol esters on the phosphorylation of endothelial plasma membranes. Lanes: A, endothelial plasma membranes phosphorylated by [τ-^{32}P]ATP (in vitro phosphorylation) in the presence of 4α-PDD (similar phosphorylation patterns were observed with plasma membranes isolated from endothelial cells metabolically labeled with $H_3{}^{32}PO_4$ [in vivo phosphorylation] in the presence of 4α-PDD [data not shown]); B, endothelial plasma membranes phosphorylated by [τ-^{32}P]ATP (in vitro phosphorylation) in the presence of TPA (similar phosphorylation patterns were observed with plasma membranes isolated from endothelial cells metabolically labeled with $H_3{}^{32}PO_4$ [in vivo phosphorylation] in the presence of TPA [data not shown]); C, phosphorylated endothelial plasma membrane proteins which were bound to a WGA-Sepharose column and eluted with 0.75 M *N*-acetylglucosamine; D, phosphorylated GP116 isolated from a rat anti-CD44-conjugated immunoaffinity column; E, nonspecific binding material (labeled with ^{32}P), using a control serum (anti-CD44 free serum) conjugated to the immunoaffinity column; F, ^{32}P-labeled endothelial plasma membrane proteins which were solubilized by Triton X-100 and bound to ankyrin-beads. Sizes are indicated in kilodaltons.

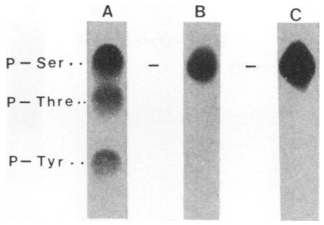

FIG. 8. Phosphoamino acid analysis of ^{32}P-labeled GP116. Lanes: A, ninhydrin staining of phosphoamino acid standards (P-Ser, phosphoserine; P-Thre, phosphothreonine; P-Tyr, phosphotyrosine); B, phosphoamino acids of phosphorylated GP116 isolated from TPA-treated endothelial plasma membranes; C, phosphoamino acids of GP116 phosphorylated by exogenously added brain PKC.

disaccharide of D-glucuronic acid and *N*-acetylgalactosamine). Other saccharides or polysaccharides such as *N*-acetyl-D-glucosamine, *N*-acetylgalactosamine, D-galactose, and D-glucuronic acid do not display any affinity for GP116 or any selective inhibition of binding of GP116 to HA (data not shown). These results suggest that binding of GP116 to HA is specific as a result of the unique repeating disaccharide structure of D-glucuronic acid or *N*-acetyl-D-glucosamine and is not simply due to the charged nature of these compounds. These results clearly indicate that GP116 is not only an endothelial cell adhesion molecule but also an HA receptor.

GP116 is a substrate for PKC. Recently, we reported that lymphoma GP85, which is also very similar or identical to CD44/Pgp-1, is specifically phosphorylated by PKC (26). Furthermore, PKC-mediated phosphorylation appears to play an important role in the overall regulation of lymphoma membrane-cytoskeleton interactions during lymphocyte receptor capping (26). In this study, we have examined the phosphorylation of endothelial cell membrane proteins by using the PKC activators TPA and its biologically inactive analog, 4α-PDD. Our results indicate that GP116 becomes phosphorylated in the presence of TPA (Fig. 7, lane B) but not in the presence of 4α-PDD (Fig. 7, lane A) under both in vivo and in vitro conditions. Phosphoamino acid analysis (Fig. 8) reveals that phosphorylation of GP116 by endogenous PKC activated by TPA occurs primarily at serine residues, that very little occurs at threonine residues, and that phosphorylation is not detectable at tyrosine residues (Fig. 8, lane B). Furthermore, phosphorylation of GP116 by exogenous brain PKC (Fig. 8, lane C) generates the same phosphoamino acid pattern as does phosphorylation by endogenous PKC (Fig. 8, lane B), with label predominantly on serine residues. Together, these findings establish that GP116 is a transmembrane glycoprotein with an intracellular domain which is a substrate for PKC.

Interaction of GP116 with ankyrin. The most extensively characterized plasma membrane-cytoskeleton organization is that which exists in erythrocytes (1) and lymphocytes (7–9, 25, 26, 31). In particular, the binding between ankyrin and certain membrane proteins, such as erythrocyte band 3 and lymphoma GP85, has been studied quite extensively (6–9, 25, 26, 31). In erythrocytes, the transmembrane glycoprotein, band 3, is known to be linked to spectrin via the linker molecule, ankyrin (2). The ankyrin binding site on the band 3 has also been localized within a 40- to 43-kDa proteolytic fragment that contains the most heavily phosphorylated region of the band 3 cytoplasmic domain. Recently, we have shown that lymphoma GP85 (CD44/Pgp-1) contains a 40-kDa peptide region that includes part of the phosphorylated cytoplasmic domain and also the ankyrin-binding site (25).

In this study, we have determined that endothelial cell GP116 also specifically binds to ankyrin attached to either Sepharose beads (Fig. 1, lane E; Fig. 7, lane F; Fig. 9, lanes A and D) or nitrocellulose sheets (Fig. 9, lane C). As a control, GP116 was preincubated with an excess amount of unlabeled ankyrin before incubation with ankyrin-beads, which completely eliminated the binding of GP116 to the ankyrin-beads (Fig. 9, lane B). When ^{32}P-labeled GP116 (Fig. 9, lane D) is partially digested by trypsin (Fig. 9, lane E), only the 20-kDa proteolytic fragment preferentially binds to ankyrin-beads (Fig. 9, lane F). In these ankyrin binding experiments, preincubation of the 20-kDa proteolytic fragment with soluble ankyrin (50 to 100 μg/ml) completely blocks binding of the 20-kDa proteolytic fragment to ankyrin-beads. This result indicates that the 20-kDa proteolytic fragment of GP116 contains at least part of GP116 ankyrin-binding site(s).

Together, our data indicate that GP116 can (i) be phosphorylated in vivo by PKC (Fig. 7, lanes A and B; Table 1, experiment C), (ii) bind to ankyrin (Fig. 9; Table 1); (iii) bind to WGA (Fig. 1, lane B; Fig. 7, lane C), and (iv) bind to HA (see above). The facts that (i) several rat anti-CD44 antibodies used in this study have been shown to interact with surface HA-binding sites (29, 30), (ii) the anti-CD44-immunoprecipitable intraluminal domain (e.g., 44 kDa) of GP116 precursor is protected from trypsin treatment (Fig. 5), and (iii) GP116 is glycosylated (Fig. 2) suggest that GP116 is a transmembrane glycoprotein. Preliminary data also indicate that GP116's WGA binding and adhesion properties (i.e., binding to HA) are abolished by treatment of intact T-lym-

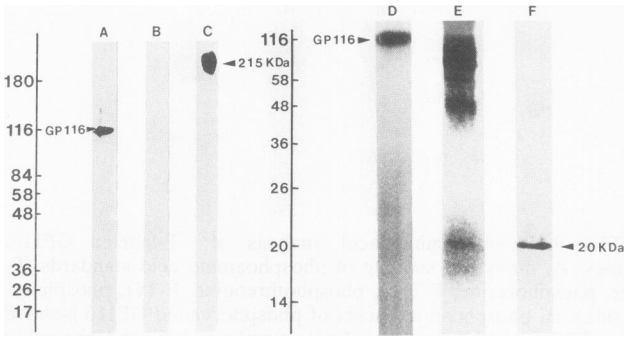

FIG. 9. Analysis of the ankyrin-binding domain of GP116. Lanes: A, ^{125}I-labeled intact GP116 which was bound to ankyrin-beads; B, ^{125}I-labeled intact GP116 which was bound to ankyrin-beads in the presence of an excess amount of ankyrin; C, ankyrin analyzed by SDS-PAGE and then Western immunoblotting and binding with ^{125}I-GP116; D, ^{32}P-labeled intact GP116 (phosphorylated by endogenous endothelial cell PKC in the presence of TPA) which was bound to ankyrin-beads; E, total trypsin-digested peptide fragments of ^{32}P-labeled GP116 before binding to ankyrin-beads; F, peptide fragments of ^{32}P-labeled GP116 bound to ankyrin-beads. (Similar results were observed with ^{32}P-labeled GP116 or trypsin-digested peptide fragments phosphorylated by brain PKC) (data not shown.) One-dimensional PAGE was done by using a linear polypeptide gradient 5 to 15% (lanes A to C) or 12% (lanes D to F) slab gel and a discontinuous buffer system as described by Laemmli (28). Sizes are indicated in kilodaltons.

phoma cells with insoluble trypsin (trypsin conjugated to Sepharose beads). Trypsin treatment does not, however, affect GP116's ankyrin-binding property (data not shown). This differential trypsin sensitivity of WGA adhesion and ankyrin binding further supports the idea that GP116 is a transmembrane glycoprotein with different domains residing on different sides of the plasma membrane.

TABLE 1. Binding of ^{125}I-labeled GP116 to ankyrin-beads[a]

Expt	Description	Radioactively labeled GP116 associated with ankyrin-beads (cpm ± range)[b]
A	GP116 phosphorylated by membrane-associated endothelial cell PKC (in vitro phosphorylation)	
	Control (no treatment)	214 ± 10
	4α-PDD treatment	229 ± 8
	TPA treatment	1,250 ± 16
B	GP116 phosphorylated by exogenously added brain PKC (in vitro phosphorylation)	
	Control (no treatment)	211 ± 9
	Brain PKC treatment	1,200 ± 14
C	GP116 phosphorylated by endogenous endothelial cell PKC (in vivo phosphorylation)	
	Control (no treatment)	203 ± 11
	4α-PDD treatment	210 ± 12
	TPA treatment	1,150 ± 18

[a] First, GP116 was phosphorylated by endothelial plasma membrane-associated PKC or brain PKC (in vitro phosphorylation) or by endogenous endothelial cell PKC in the presence of TPA or 4α-PDD (in vivo phosphorylation) as described in Materials and Methods. Phosphorylated GP116 was subsequently labeled with ^{125}I, using Na^{125}I and the Iodo-Gen method (17). ^{125}I-GP116 was then used in the ankyrin-bead binding assay as described in Materials and Methods. As a control, preincubation of GP116 with soluble ankyrin (50 to 100 μg/ml) completely blocks binding of GP116 to ankyrin-beads.

[b] Average of three to five experiments.

TABLE 2. Phosphorylation of GP116 by endothelial cell PKC and brain PKC[a]

Expt	Description	Phosphorylation (mol of P_i/mol of GP116)
A	GP116 phosphorylated by membrane-associated endothelial cell PKC	
	Control (no treatment)	0.205
	4α-PDD treatment	0.213
	TPA treatment	1.260
B	GP116 phosphorylated by brain PKC	
	Control (no treatment)	0.208
	Brain PKC treatment	1.250

[a] Endothelial plasma membrane fraction or brain PKC was used as a PKC source to phosphorylate GP116 as described in Materials and Methods.

Finally, we have examined the question of whether the observed membrane-cytoskeleton interaction is directly regulated by protein phosphorylation. Phosphorylation of membrane proteins or cytoskeletal proteins by PKC has been shown to be involved in the modulation of certain membrane-cytoskeleton interactions (26). The results of our study clearly indicate that GP116 phosphorylation can proceed from a low degree of phosphorylation to a much higher level by treatment with either endogenous PKC (activated by TPA) (Table 2, experiment A; Fig. 7, lanes A and B; Fig. 8, lane B) or exogenous PKC (Table 2, experiment B; Fig. 8, lane C) in a manner similar to that described for GP85 (CD44) of mouse T-lymphoma cells (26). Most importantly, phosphorylation of GP116 by PKC (either in vivo or in vitro) significantly enhances binding (at least a fivefold increase) of GP116 to ankyrin-beads (Table 1). These findings strongly suggest that PKC plays a central role in the regulation of endothelial GP116-cytoskeleton interactions.

In conclusion, we believe that GP116 is a CD44-like protein in endothelial cells. The evidence is summarized as follows: (i) it displays immunological cross-reactivity with a panel of CD44 antibodies (Fig. 1, lane C; Fig. 3, lanes A to C; Fig. 7, lane D), (ii) it shares some peptide similarity with CD44 (Fig. 4), (iii) it has a similar 52-kDa precursor molecule which is also a biosynthetic precursor of CD44 (Fig. 5), (iv) it binds specifically to HA (see above), (v) it is phosphorylated in the presence of phorbol ester (TPA) (Fig. 7, lanes A and B), (vi) it is phosphorylated primarily at serine residues (Fig. 8), and (vii) it can bind directly to ankyrin (Fig. 9). The fact that PKC-mediated phosphorylation of GP116 enhances its interaction with ankyrin (Table 1) suggests that phosphorylation of the transmembrane glycoprotein GP116 (a CD44-like molecule) by PKC is important for GP116-ankyrin interaction during endothelial cell adhesion.

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