Albumin interacts specifically with a 60-kDa microvascular endothelial glycoprotein
(receptors/lectins/capillary permeability/glycocalyx/plasmalemmal proteins)

JAN E. SCHNITZER, WILLIAM W. CARLEY, AND GEORGE E. PALADE
Department of Cell Biology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510

Contributed by George E. Palade, May 19, 1988

ABSTRACT Confluent monolayers of microvascular endothelial cells, derived from the rat epididymal fat pad and grown in culture, were radiiodinated by using the lactoperoxidase method. Their radiiodinated surface polypeptides were detected by NaDodSO₄/PAGE (followed by autoradiography) and were characterized by both lectin affinity chromatography and protease digestion to identify the proteins involved in albumin binding. All detected polypeptides were sensitive to Pronase digestion, whereas several polypeptides were resistant to trypsin. Pronase treatment of the cell monolayer significantly reduced the specific binding of radiiodinated rat serum albumin, but trypsin digestion did not. Limax flavidus, Ricinus communis, and Triticum vulgaris agglutinins competed significantly with radiiodinated rat serum albumin binding, whereas other lectins did not. A single 60-kDa glycoprotein was precipitated in common by these three lectins and was trypsin-resistant and Pronase-sensitive. Rat serum albumin affinity chromatography columns weakly but specifically bound a 60-kDa polypeptide from cell lysates derived from radiiodinated cell monolayers. These findings indicate that the 60-kDa glycoprotein is directly involved in a specific interaction of albumin with the cultured microvascular endothelial cells used in these experiments.

The attenuated layer of endothelial cells lining the blood vessels forms the critical barrier controlling the exchange of molecules between the blood and the interstitial fluid. The ability of the endothelium to act both as a passive, albumin-modified barrier to transvascular exchange (1, 2) and as a specific receptor-mediated translocutor of molecules [i.e., insulin (3), transferrin (4, 5), and low density lipoprotein (6)] is dependent on interactions occurring at its luminal surface. Through its interaction with the endothelium, albumin has several important vascular functions. As the major blood protein, it is the principal determinant of the oncotic pressure of the plasma and thereby strongly influences transendothelial fluxes of water and small solutes. In addition, its interaction with the endothelial glycocalyx (2, 7, 8) apparently creates a perselective barrier that limits the transendothelial passage of many molecules (1, 9). Upon removal of albumin from vascular perfusates, for instance, apparent solute and water permeabilities increase significantly while osmotic reflection coefficients decrease in both individually perfused capillaries (1) and whole organ preparations (2, 10). Albumin also acts as a carrier of amino acids, heavy metal ions, fatty acids, steroids, drugs, hormones, and bilirubin (11, 12), an activity that involves its own transport across the endothelium. Once transported to the interstitial fluid, albumin is actively involved in the presentation of important ligands to various cells, including hepatocytes (13), myocytes (14), and adipocytes (15). In addition, albumin interacting with the endothelial glycocalyx may reduce platelet and erythrocyte adhesion (15) and restrict the surface binding of other plasma proteins (9, 16).

At present there is little general agreement as to the pathways and mechanisms of transendothelial transport of albumin and other macromolecules. Physiological studies indicate that albumin and other macromolecules cross the endothelium through a hydraulically conductive pathway, modeled simply as pores or filtration slits usually assumed to be located along intracellular junctions (1). However, data from different laboratories show that insulin (3), low density lipoprotein (6), and transferrin (4, 5) are transported across the endothelium by a vesicular, receptor-mediated process, and recent work indicates that monomeric albumin traverses microvascular myocardial endothelium by way of plasmalemmal vesicles (8), a conclusion supported by earlier work on other microvascular beds (2, 17). Moreover, receptor-mediated transcytosis of albumin across the endothelium has been proposed based on the apparent preferential association of albumin-gold complexes with plasmalemmal vesicles (18). Albumin receptors also may exist on other cell types (13, 14). For all of these reasons, it is important to define not only the physiological and structural parameters of albumin transport and its effect on the transport of other molecules but also the molecular interactions involved in these processes.

Albumin has been localized immunocytochemically both in situ and in culture to the endothelial glycalyx of the plasmalemma proper (2, 7, 8), plasmalemmal vesicles (2, 7, 8, 18), and in some cases coated pits (7). The binding of 125I-labeled albumin to cultured microvascular endothelial cells is specific, rapid, saturable, reversible, dependent on incubation time, cell type, and cell number, and also negatively cooperative in nature (7). It is competitively inhibited by unlabeled albumin, and it is apparently polarized to the substrate-free, outer surface of cell monolayers (7). It has an apparent affinity constant of 1 ng/ml and a maximum surface binding concentration of 90 ng/cm² (7); both values are consistent with the data obtained in studies of albumin binding to adipocytes and hepatocytes (13). Chemical modification of arginine (but not lysine) residues of albumin significantly reduces binding to the endothelium (19) and increases transcapillary water fluxes (20). Electrostatic and steric partition theory applied to transendothelial transport indicates that the concentration of albumin detected within the glycocalyx is sufficient to account for the observed changes in osmotic reflection coefficients (9).

In this study, we begin to identify and characterize both the plasmalemmal proteins of a widely used microvascular endothelial cell culture system and the potential specific mo...
lecular interaction of these proteins with albumin. Lectin binding and protease treatment have provided a sorting scheme that has led to the identification of a trypsin-resistant plasmalemmal glycoprotein of 60 kDa (gp60) that interacts specifically with albumin. Lectin binding to gp60 decreases significantly the specific binding of albumin to the endothelium.

METHODS

Endothelial Cells and Cultures. Microvascular endothelial cells, isolated from rat epididymal fat pads (RFC) on a Percoll gradient by J. Madri as previously described (21), were grown and plated to achieve confluent monolayers as in ref. 7. The endothelial origin of the cultured cells was tested periodically by using surface immunostaining for angiotensin-converting enzyme or uptake of acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-3,3',3'-tetramethylindocar-bocyanine perchlorate as in ref. 7.

Lectin Fluorescence Microscopy. RFC cells, grown to confluency in 8-well slide chambers, were washed twice at room temperature with 300 μl of phosphate-buffered saline (PBS) per well and were incubated for 1 hr with 200 μl of PBS containing a single tetramethylrhodamine isothiocyanate-la-beled lectin at 10 μg/ml. The lectins used, their abbreviations, and nominal specificities as well as the cognate haptons used (in parentheses) are as follows: concanavalin A (Con A; α-D- mannose > β-D-glucose (glucose)); Limax flavus agglutinin (LFA; N-acetylneuraminic acid (N-acetylneuraminic acid)); Ricinus communis agglutinin (RCA; β-D-galactose > α-D-galactose >> N-acetylgalactosamine (galactose)); Glycine max agglutinin [SBA; N-acetyl-α-galactosamine and N-acetyl-β-galactosamine >> α- and β-galactose (N-acetylgalactosamine)]; Ulex europaeus agglutinin [UEA; α-L-fucose (fucose)]; and Triticum vulgaris agglutinin [WGA; N-acetyl-β-glucosaminyl-(1 → 4)N-acetyl-β-glucosamine >> N-acetyl-β-glucosamine > N-acetylneuraminic acid (N-acetylneuraminic acid)]. After incubation, the cells were washed twice for 1 min in PBS (300 μl); the wells and gasket were removed; and the slide was mounted with a coverslip and was examined and photographed with a Zeiss fluorescence microscope set for rhodamine excitation/emission viewing.

Radioligand Assay. Single monolayers were washed at 37°C once for 15 min and twice for 1 min with 1 ml of Dulbecco’s modified essential medium (DMEM) followed by three washes (1 min each) with 1 ml of PBS at room temperature. Each monolayer was reacted at room temperature with 1 ml of PBS containing 0.1 mM NaI, 60 μg of lactoperoxidase (Sigma), and 15-20 ng (0.3-0.4 μCi; 1 Ci = 37 GBq) of Na125I (Amersham). Initially and at four successive 3-min intervals, 10 μl of 0.5 mM H2O2 in double-distilled water was added to each dish followed by mild circular agitation (10 sec). After 15 min, the reaction was stopped by aspirating the reaction mixture from the dishes and washing the cell monolayers three times (1 min each) with 1 ml of PBS.

NaDodSO4/PAGE. For gel electrophoresis, cell lysate samples (see figure legends for lysis conditions) were mixed 2:1 (vol/vol) with solubilization buffer containing 0.5 M Tris-HCl (pH 6.8), 9% (wt/vol) NaDodSO4, 3.6% (vol/vol) 2-mercaptoethanol, 6 M urea, and 6 M EDTA in double-distilled water. They were incubated at 80°C for 10 min and electrophoresed on 1.5-mm slab gels cast as previously described (22) with a 3% stacking gel and a 5-15% gradient separating gel. Gels were fixed, stained, dried, and processed for autoradiography at ~70°C with Kodak X-Omat AR film.

Lectin Affinity Chromatography. Biotinylated LFA, Con A, WGA, RCA, or SBA (E-Y Laboratories, San Mateo, CA) [50 μg in 100 μl of 0.68 mM CaCl2 in PBS at pH 7.4 (PBS/CA)] were each mixed with 20 μl of avidin-agarose beads (E-Y Laboratories) and were incubated with agitation on a Nutator for 1 hr at room temperature. The beads were washed three times (1 min each) with 1 ml of wash buffer (1% Triton X-100/0.2% NaDodSO4 in PBS) at 4°C, incubated at 4°C for 1 hr with 50 μl of radioidinated cell lysate diluted with 50 μl PBS/Ca, and washed twice (1 min each) at 4°C with 1 ml of wash buffer followed by a 1-min wash with 1 ml of PBS. The glycoconjugates bound to the lectin beads were solubilized with 50 μl of solubilization buffer, heated to 80°C for 10 min, and then analyzed by NaDodSO4/PAGE followed by autoradiography.

RESULTS

In our previous work (7), we showed that cultured endothelial cells specifically bind RSA. Binding parameters were defined by biochemical radioassays, and the binding sites were localized by immunocytochemistry to the free side of the endothelium. On the basis of these (7) and other (5, 6, 18) results, we assumed that proteins, primarily glycoproteins, of the luminal plasmalemma are involved in albumin binding. To test this assumption, we labeled by radioiodination the plasmalemmal proteins of intact cultured endothelial cells and then sorted out the labeled proteins in two steps: the first involved protease treatment and the second involved lectin binding. In parallel experiments, albumin binding to the cells was tested either after protease treatment or in the presence of various lectins.

Radioiodinated Plasmalemmal Proteins. Lactoperoxidase-catalyzed radioiodination of intact cells, followed by cell lysis and lysate processing through NaDodSO4/PAGE and autoradiography, revealed the presence of ~10 125I-labeled protein bands ranging in molecular mass from 30 to 300 kDa (Fig. 1). About 10% of the loaded radioactivity remained on top of the gel or hardly penetrated the running gel.

Sorting by Protease Treatment. Pronase treatment of the cells after radioiodination resulted in the removal of all radioactive bands except one of ~68 kDa, which by comparison with the corresponding controls (Fig. 1) appeared to be a degradation product of labeled proteins of molecular mass >100 kDa. Treatment of radioidinated cells with trypsin removed practically all bands >110 kDa, reduced the amount of radioactive protein present in aggregates at the top of the gel, and left protein bands of 60, 56, 45, and 35 kDa. The results strongly suggested that all the 125I radioactivity was in the ectodomains of the plasmalemmal proteins, that all of these ectodomains were Pronase-sensitive, and that some of them were labeled.

Sorting by Lectins. In a preliminary survey, lectin fluorescence microscopy was used to detect specific monosaccharide residues within the glyocalyx of confluent microvascular endothelium in culture (Fig. 2). WGA gave a diffuse positive reaction over a fine punctate staining. LFA generated a weak diffuse reaction. Con A and RCA gave diffuse positive reactions reinforced at the level of cell margins and surface foldings. SBA produced a coarse and unevenly
Fig. 1. Autoradiogram of NaDodSO4/PAGE gel of radioiodinated endothelial surface proteins. Confluent monolayers of RFC cells were labeled with 125I. One monolayer was immediately solubilized in 300 μl of 5% Triton X-100/1% NaDodSO4 in PBS (Control I) while the others were incubated at 37°C for 5 min with 1 ml of one the following: 2 mM EDTA in PBS; 0.25% trypsin with 2 mM EDTA in PBS; or Pronase [from Streptomyces griseus type XIV (Sigma)] at 1 mg/ml with 2 mM EDTA in PBS (2 mM EDTA in PBS was used for uniformity and to facilitate detachment and minimize cell damage upon scraping). Since both protease digestions disrupted the cell monolayer, the samples incubated with trypsin, Pronase, and EDTA were scraped from the dish and pelleted by centrifugation. The pellets were washed three times (1 min) with 1 ml of DMEM at 4°C by resuspension and sedimentation, solubilized, and then processed for NaDodSO4/PAGE followed by autoradiography. The open arrowhead indicates the 60-kDa protein band. The same volume of cell lysate was loaded onto each lane of the gel. The molecular mass standards used were myosin (205 kDa), β-galactoside (116 kDa), phosphorylase B (97 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa).

distributed punctate staining, and UEA gave a negative reaction (not shown).

Based on these results, lysates of radioiodinated RFC cells were incubated with immobilized WGA, LFA, RCA, Con A, and SBA, and the ensuing lectin-binding glycoproteins were identified by NaDodSO4/PAGE and autoradiography (Fig. 3). The results of these experiments showed that (i) as a group, the lectins used bound (and precipitated) only part of the radiiodinated plasmalemmal proteins (compare Fig. 1 to Fig. 3) and (ii) the patterns of glycoproteins were different qualitatively and quantitatively from one lectin or another. For instance, SBA precipitated only a single 74-kDa protein; LFA bound one major protein of 60 kDa; RCA recognized one major (60 kDa) and one minor (74 kDa) glycoprotein; WGA strongly precipitated two major proteins of 60 and 74 kDa; Con A precipitated the most extensive and diffuse collection of surface glycoproteins, including a diffuse band centered at 74 kDa (possibly two bands of 74 and 78 kDa); WGA, LFA, RCA, and Con A all precipitated to various minor degrees glycoproteins of 56, 100, 120, 145, 165, 190, and 230 kDa (see longer exposure lanes). In all cases, radioactive aggregates were found at the top of the running gels; LFA, RCA, and WGA precipitated about 6-fold greater radioactivity than did Con A and SBA.

Effects of Protease Treatment on Albumin Binding. Since Pronase and trypsin differentially remove the ectodomains of plasmalemmal proteins, we decided to investigate how these enzymes affect albumin binding to intact RFC cells. 125I-RSA binding assays (Fig. 4) showed that Pronase treatment resulted in a drastic reduction of 82 ± 11% of specific 125I-RSA binding, whereas trypsin appeared to be essentially ineffective (14 ± 6%). These findings implicated Pronase-sensitive but trypsin-resistant plasmalemmal proteins as potential candidates for albumin binding sites.

Effects of Lectin Competition on Albumin Binding. To advance the identification of such glycoproteins, we carried out 125I-RSA binding assays in the presence of each of the
Cell Biology: Schnitzer et al.

**Fig. 4.** Specific $^{125}$I-RSA binding to endothelial cells after enzymatic digestion of their glycoalyx. Confluent RFC monolayers were treated with either PBS, EDTA, trypsin, or Pronase as described in Fig. 1 and incubated for 20 min with agitation in 1 ml of $^{125}$I-RSA (0.6 mg/ml) in DMEM. At the end of the incubation, the cells were washed three times (30 sec each) with 1 ml of DMEM (by using resuspension and sedimentation for the cell suspensions) and were finally counted for radioactivity. The EDTA-treated cells were a second control, which provided correction for the change in protocol with trypsin and Pronase digestion and indicated that Pronase digestion of the cell surface greatly reduced $^{125}$I-RSA binding, whereas trypsin digestion did not change it. Specific binding was defined as $^{125}$I-RAA associated with the cells that could be blocked by simultaneous incubation with >100-fold excess of unlabeled RSA (100 mg/ml). All results were normalized to the mean value of albumin bound for the control group (32.5 ± 6.0 ng/cm$^2$), which was considered 100% binding. The mean ± SD for each group is given. Unpaired r tests were used to compare the results. *, Statistically significant difference from the two control groups and the trypsin digestion ($P > 0.05$); $N$, number of dishes assayed. The Tryp + RCA bar shows the albumin bound to trypsin-digested RFC cells in the presence of RCA at 50 μg/ml; albumin binding is reduced by ≈50%.

**Fig. 5.** Competition of various lectins with the binding of $^{125}$I-RSA to RFC cell monolayers. The usual $^{125}$I-RSA binding protocol was followed by using $^{125}$I-RSA (0.6 mg/ml) in DMEM or PBS at 4°C either alone or premixed with 50 μg of either the lectin indicated (stippled bar) or the lectin plus its specific hapten monosaccharide (0.1 M) (hatched bar) as given in Methods. All results are normalized and presented as described in Fig. 4.* Statistically significant difference from the observed binding of $^{125}$I-RSA from both control groups: one without lectin present (CONTROL) and one with both lectin and its specific cognate hapten monosaccharide present ($P > 0.99$); **, $P > 0.99$ for the CONTROL and $P > 0.90$ for the lectin plus hapten (n = 4 for lectin alone; n = 2 for lectin plus hapten).

**DISCUSSION**

In this study we have identified several microvascular endothelial plasmalemmal proteins and characterized them according to their susceptibility to protease digestion and their

**Fig. 6.** Autoradiogram of NaDodSO$_4$/PAGE gel of radioiodinated endothelial proteins after affinity chromatography on immunobilized RSA. Beads conjugated with RSA were incubated for 1 hr at 4°C with the solubilized, radioiodinated cell lysate either alone or in the presence of RSA (1 mg/ml) or gelatin (1 mg/ml). After three washes (1 min each) with 1 ml of PBS, the bound complexes were solubilized with solubilization buffer and processed through NaDodSO$_4$/PAGE and autoradiography (72-day exposure). Molecular mass markers (in kDa) are indicated at left.

These results suggested that certain lectins interfered with albumin binding to the ectodomains of endothelial plasmalemmal proteins, presumably by covering part of the latter's binding sites or by otherwise preventing access to these sites. The binding experiments indicate that the most likely candidate for albumin binding should bind WGA, LFA, and RCA and be trypsin-resistant. As indicated in Figs. 1 and 3, gp60 apparently fulfills these requirements.

**RSA Precipitation of gp60.** Affinity chromatography with RSA conjugated to Sepharose beads (see Methods) specifically detected a single protein of 60 kDa in the cell lysates (Fig. 6). RSA added to the cell lysate before RSA chromatography eliminated the detection of this protein, whereas gelatin did not. Under these solubilization conditions, the interaction is very weak as indicated by the long exposure time. The amount of radiolabeled protein detected at the top of the separating gel does not change appreciably in the presence of RSA or gelatin.

**Testing the gp60–Albumin Interaction Hypothesis.** A comparison of Figs. 1 and 3 pointed to a single membrane glycoprotein, gp60, that is trypsin-resistant and binds the three lectins that compete with albumin binding. This glycoprotein does not bind the other lectins tested. The other trypsin-resistant glycoproteins do not bind or bind less effectively to the lectins that inhibit specific $^{125}$I-RSA binding. To test the assumption that gp60 is a likely candidate, RCA was used to compete, after trypsin digestion, with $^{125}$I-RSA binding to RFC cells and to precipitate from the lysates of such cells trypsin-resistant plasmalemmal proteins. The results showed that, under these conditions, RCA inhibited by 50% $^{125}$I-RSA binding to the cells (see Fig. 4) and complexed a single glycoprotein of apparent molecular mass of 60 kDa from cell lysates (Fig. 7). In the presence of galactose, there was no formation of RCA-gp60 complexes. RCA competed with albumin binding both before (Fig. 4) and after (Fig. 5) trypsin digestion. It also precipitated gp60 both before (Fig. 3) and after (Fig. 7) trypsin treatment; gp60 was the only glycoprotein precipitated after digestion. On the basis of these results, we conclude that gp60 apparently is one of the molecules, perhaps a major molecule, involved in albumin binding to the exposed plasmalemma of RFC cells in culture.
ability to bind various lectins. In agreement with past investigations done in situ on various different rodent vascular beds (23–25), mammamys, glucosyl, galactosyl, N-acetylglucosaminyl, N-acetylgalactosaminyl, and sialyl residues but not fucosyl residues were identified as constituents of the glycocalyx in rat aortic endothelial cells grown in culture with a surface carbohydrate composition similar to that found in situ. In addition, we have identified for the first time specific lectin binding endothelial glycoproteins (see Fig. 3), which may represent the lectin receptors seen in situ.

In this study, we were primarily interested in the interaction of albumin with the plasmalemmal proteins of the endothelial cells. Our results establish that specific albumin binding to cultured monolayers of RFC cells is differentially sensitive to proteinase digestion and the presence of different lectins in the binding assays. Binding is Pronase-sensitive but trypsin-resistant. These findings narrow the number of likely candidates for albumin binding sites to a few plasmalemmal surface proteins of molecular mass <100 kDa. Lectin inhibition of albumin binding coupled with the precipitation of endothelial glycoproteins by lectins eliminates some of these candidates and implicates a gp60 as a binding site for albumin. The assumption was tested further by treating RFC cell monolayers with trypsin and by using RCA to show both inhibition of specific 125I-RSA binding to these cells (Fig. 4) and complex formation with gp60 (Fig. 7).

On the basis of the evidence provided by these protease/lectin sorting experiments, it appears that gp60 is an albumin binding plasmalemmal protein of the microvascular endothelial cells used in this study. The weak but specific binding of a 60-kDa radioiodinated protein to immobilized RSA provides direct support for this interaction. Evidence obtained by cross-linking albumin to the plasmalemmal proteins of cultured endothelial cells also implicates a 60-kDa polypeptide (26).

In all our experiments, about 10% of the radioactivity imparted by radiiodination of endothelial surface proteins remained in the stacking gel or hardly penetrated the running gel. Some of the aggregated proteins were trypsin-resistant and bound the same lectins as did gp60. These aggregates deserve, therefore, to be further investigated to find out if they are involved in the albumin–endothelial surface interaction and if they comprise gp60 or some other albumin-binding protein(s). The behavior of these aggregates in NaDodSO4/PAGE suggests that they may include proteoglycans known to be produced by many endothelial cells. Since albumin interacts with many different molecules, including lipids, other groups of molecules should be considered (e.g., either glycolipids, polar lipids, or surface proteins not solubilized by the detergents used or not labeled by the radiiodination procedure applied).

Finally the kinetics of albumin binding to intact RFC cells show negative cooperativity but do not exclude the existence of albumin-binding sites of different affinities (7). Moreover, immunocytochemical studies of albumin binding to the endothelium reveal the involvement of the plasmalemma proper as well as plasmalemmal vesicles (2, 7, 8). Plasmalemmal vesicles and possibly transendothelial channels are involved in the transendothelial transport of albumin (8), perhaps by means of receptor-mediated transcytosis (18). Albumin receptors located in these vesicles may be different from those distributed more widely on the endothelial plasmalemma, which may generate the permissive barrier at the level of the glycocalyx (1, 2, 9). Albumin binding within vesicles also may restrict sterically and electrostatically the transendothelial transport of other plasma molecules (9). Interestingly, lectin binding experiments done in other vascular beds in situ show that vesicles and possibly transendothelial channels may contain sialyl and galactosyl residues (24, 25). gp60 also contains these monosaccharide residues. It remains to be seen whether other proteins in addition to gp60 are involved in the generation of all of these effects.

Note Added in Proof. A paper identifying albumin binding proteins of smaller molecular mass than gp60 was recently published by Ghinea et al. (27).

We are grateful for the technical assistance of Rita Palmarozza (tissue culture) and Ann Curley-Whitehouse (photography). This work was supported by National Institutes of Health Grant HL17080 and a gift from RJR Nabisco, Inc.