gp60 is an albumin-binding glycoprotein expressed by continuous endothelium involved in albumin transcytosis

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Schnitzer, Jan E. gp60 is an albumin-binding glycoprotein expressed by continuous endothelium involved in albumin transcytosis. *Am. J. Physiol.* 262 (*Heart Circ. Physiol.* 31): H246–H254, 1992.—Albumin reduces capillary permeability and acts as a carrier for various small molecules. Recently, we identified a 60-kDa sialoglycoprotein (gp60) on the surface of cultured rat microvascular endothelial cells (MEC) that binds albumin and antiglycophorin serum (α-gp). We verified that α-gp recognizes the albumin-binding gp60 by affinity, purifying proteins from MEC extracts using immobilized albumin. gp60 was immunoblotted with α-gp only when the MEC extract was reacted with albumin and not in controls. We immunoprecipitated gp60 from biosynthetically radiolabeled MEC lysates and from extracts containing endothelial surface proteins of isolated rat hearts that were radioiodinated in situ. gp60 was immunoblotted selectively in rat tissue microvascular beds lined with continuous endothelium (heart, lung, diaphragm, fat, skeletal muscle, mesentery, and duodenal muscularis but not cortical brain) and not those exclusively lined with fenestrated or sinusoidal endothelium (adrenal, pancreas, liver, and small intestinal mucosa). MEC isolated from rat heart, lung, and epididymal fat pad expressed gp60 and bound albumin, whereas various nonendothelial cells and brain-derived MEC did not. gp60 is an albumin-binding glycoprotein expressed specifically on the surface of continuous endothelium that binds albumin apparently not only to initiate its transcytosis via plasmalemmal vesicles but also to increase capillary permselectivity.

rat; biological transport; capillary permeability; membrane receptors; membrane proteins; sialoglycoproteins; receptor-mediated transport; vesicular transport; plasmalemmal vesicles; glycocalyx

A PRIMARY FUNCTION of the attenuated layer of vascular endothelium is to act as a barrier to the transvascular transport of plasma molecules in many tissues. The selectivity of the endothelial barrier varies in different vascular beds and is strongly dependent on the structure and type of endothelium lining the microvasculature (29). Several pathways exist for the transport of plasma molecules across continuous endothelium: *1*) intercellular junctions are highly regulated structures that form the paracellular pathway for the passive, pressure-driven filtration of water and small solutes; *2*) plasmalemmal vesicles transcytose plasma macromolecules, apparently by shuttling their contents adsorbed from blood from the luminal to antiluminal aspect of the endothelium (16); and *3*) transendothelial channels, which may form transiently by the fusion of two or more plasmalemmal vesicles, may provide a direct conduit for the exchange of both small and large plasma molecules (31). Recently, it has become clear that capillary permeability in many vascular beds is also dependent on the interaction of these transendothelial transport pathways with the multifunctional plasma protein, albumin (3, 6, 11–14, 19). Albumin binds to the luminal glycocalyx of continuous endothelium (10, 15, 19, 22), and its binding within transport pathways, such as plasmalemmal vesicles and the introit of intercellular junctions, apparently forms a molecular filter within these pathways (3) that can electrostatically (20) and sterically (3, 21) restrict the transport of water, small solutes, and macromolecules across the microvascular wall (6, 11–14, 19). In addition, albumin is transcytosed across vascular endothelium via plasmalemmal vesicles (10, 15, 19) and acts as a carrier for small ligands bound to it, such as fatty acids (5). This apparent receptor-mediated transcytosis of albumin appears to occur selectively in certain tissues with vascular beds lined with continuous endothelium (10, 28) and is influenced greatly by the ligands bound to albumin (5).

The binding of albumin to cultured microvascular endothelium has been quantitated and immunolocalized to the surface of cultured rat microvascular endothelial monolayers (22). Specific albumin binding was shown to be saturable, reversible, competible, dependent on cell type and cell number and to have a negative cooperativity in nature (22). Albumin binding was sensitive to pronase but not to trypsin digestion of the cell surface and was inhibited significantly by the presence of *Limax flavus* (LFA), *Ricinus communis* (RCA), and *Triticum vulgare* (wheat germ; WGA) agglutinins but not several other lectins (23). Recently, a group of rat endothelial plasmalemmal sialoglycoproteins has been identified both in situ and in culture (25). One of these sialoglycoproteins, called gp60, was identified as an albumin-binding protein because it *1*) interacts with albumin conjugated to beads (23); *2*) binds RCA, LFA, and WGA but not other lectins (23, 25, 26); and *3*) is sensitive to pronase and sialidase but not to trypsin digestion (23, 25). Another laboratory has also identified gp60 as one of three major albumin-binding proteins (8, 9). The structural and functional relationship of these albumin-binding proteins to one another is unknown. Further characterization of gp60 showed that it apparently contains *O*-linked but not *N*-linked glycans (25) and may also be antigenically related to another sialoglycoprotein, namely glycophorin (26). Polyclonal antiserum (α-gp) raised against purified mouse glycophorin gp3 recognized a 60-kDa WGA- and RCA-binding sialoglycoprotein on the surface of cultured rat microvascular endothelial cells (26). This apparent recognition of gp60 by α-gp was inhibited in the presence of murine glycophorins.

In this study, the specific interaction of gp60 with albumin and with α-gp serum is demonstrated. Then, α-gp is used as a probe for gp60. Because albumin binding and transcytosis via plasmalemmal vesicles are not observed in the endothelium lining the vasculature of many tissues, tissue-specific expression of gp60 should be ex-

pected if gp60 does indeed function as a physiologically significant albumin-binding protein. Therefore various rat tissue and cell extracts were tested using α-gp for the presence of gp60. In addition, microvascular endothelial cells have been isolated from several organs and grown in culture to compare both gp60 expression and albumin surface binding. These experiments demonstrate *1*) the specific expression of gp60 only by those cultured endothelial cells that bind albumin and not various other cells, *2*) the presence of gp60 on the surface of vascular endothelium both in situ and in culture, and *3*) the selective distribution of gp60 in rat tissues with vascular beds lined with continuous endothelium that transcytose albumin via plasmalemmal vesicles.

METHODS

Isolation and growth of endothelial cells in culture. Male albino rats (Sprague-Dawley, 200–250 g) were anesthetized with ether. The heart, lungs, and cortical brain were surgically removed from three to five rats, were submerged in cold Dulbecco's modified Eagle's medium (DMEM) supplemented with 15–20% fetal calf serum (FCS) (DMEM+), and each was minced in a vial using small sterile surgical scissors. For the heart, only myocardium was used after careful excision of the epi- and endocardium. For the lung, only peripheral regions of the lobes were used. Both of these excision procedures are designed to eliminate obvious large arterial and venous segments of the vasculature and to increase the probability for isolation of microvascular endothelium. After centrifugation for 5 min at 1,000 *g* at 4°C, the pellet was resuspended in 2 mg/ml of collagenase (from *Clostridium histolyticum*, type II, Sigma) in DMEM+ at 37°C using five times the tissue volume. After a 1-h incubation, the suspension was poured over a sterile Nitex monoscreen cloth (no. 3–112–40xx; Tetko, Briar Cliff Manor, NY) to filter out tissue clumps. The filtrate was centrifuged for 5 min at 1,000 *g*, and the pellet was resuspended in DMEM+ containing 20 μg/ml of heparin, 100 U/ml of penicillin G, 100 μg/ml of streptomycin sulfate, and 10% bovine aortic endothelium-conditioned media. The cells were plated onto T-25 flasks (Corning, Corning, NY) for culture at 37°C with 5% CO_2 in air. After several days when confluency was reached, the cells were examined and sorted on a fluorescent-activated cell sorter using uptake of acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI-AcLDL) as in Refs. 22 and 30. Madin-Darby kidney cells (MDCK) cells were used as a negative control. Microvascular endothelial cells that exhibited uptake of DiI-AcLDL and the mixture of other cells in the heart and lung preparations that did not internalize DiI-AcLDL were replated separately and grown in culture using the above media (without heparin). After 24 h, this medium was replaced for all future culturing with DMEM+ for the nonendothelial cells and with DMEM+ with retinal-derived growth factor for the endothelial cells. Microvascular endothelial cells isolated from rat epididymal fat pads (RFC) were grown and plated as previously described (22).

Immunoblotting of total cell lysate from cultured cells. After washing the confluent cell monolayers, their proteins were solubilized directly with cold solubilization buffer (SB) containing 0.17 M tris(hydroxymethyl)aminomethane (Tris)·HCl (pH 6.8), 3% (wt/vol) sodium dodecyl sulfate (SDS), 1.2% (vol/vol) β-mercaptoethanol, 2 M urea, and 3 mM EDTA in double-distilled water as described previously (25). After incubation in boiling water for 10 min, a lysate volume equivalent of 10^6 cells was processed for SDS-polyacrylamide gel electrophoresis (PAGE), and the separated proteins were electrotransferred onto Immobilon filters (Millipore, Bedford, MA) as in Refs. 25 and 26. Strips of these filters were immunoblotted with rabbit serum, and any bound immunoglobulin G (IgG) was detected using anti-rabbit IgG antibodies conjugated to alkaline phosphatase as in Ref. 26.

Immunoprecipitation of gp60 radiolabeled biosynthetically using tritiated sugars. About 5×10^6 RFC cells were plated onto two T-75 flasks. After 1.5 h, 1.0 mCi of [2,6-^{3}H]mannose (45 Ci/mmol; Amersham, Arlington Heights, IL) mixed in 3 ml of DMEM+ was added to one flask of cells or 1.0 mCi of [6-^{3}H]galactose (20 Ci/mmol; ICN, Costa Mesa, CA) combined with 1 mCi of [6-^{3}H]glucosamine (27 Ci/mmol; ICN) mixed in 3 ml of DMEM+ was added to another flask of cells. After 3 days when the cell monolayer reached full confluency, the cells were washed with 10 ml of DMEM (3 times, 1 min) at 4°C, lysed with 5% Triton X-100 and 1% SDS in phosphate-buffered saline (PBS), and finally scraped from the flask. After a 10-min spin at 4°C (1,000 *g*), the supernatant was used for immunoprecipitations overnight with α-gp as described in Ref. 25. The precipitates were analyzed by SDS-PAGE and were visualized by fluorography as per Ref. 1.

Immunoprecipitation of gp60 radioiodinated in situ. The endothelial luminal surface proteins of the heart vasculature were radioiodinated in situ using Na^{125}I, and microspheres were covalently coated with lactoperoxidase and glucose oxidase as described previously (25). Proteins were extracted from 200 mg of heart tissue by mincing the tissue in 500 μl of 5% Triton X-100 and 1% SDS in PBS at 4°C. The lysate was centrifuged at 13,000 *g* for 1 min at 4°C, and 100 μl of the supernatant (tissue extract) was mixed overnight at 4°C with 380 μl of PBS and 20 μl of rabbit antiserum. The ensuing immune complexes were then precipitated with protein A conjugated to Sepharose beads as in Ref. 25. The proteins bound to the beads were solubilized directly with 150 μl of cold SB, kept in boiling water for 10 min, and analyzed by SDS-PAGE followed by autoradiography as described previously (25).

Immunoblotting of tissue extracts. Male albino rats (Sprague-Dawley, 200–250 g) were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (33 mg/kg). The chest was opened through a median sternotomy. Through a needle inserted into the left ventricle and after the right atrium was cut for outflow purposes, the vasculature was perfused with DMEM (Irvine Scientific, Irvine, CA) at a mean pressure of 60 mmHg first for 5 min at 37°C and then for 10 min at 10°C. The heart, liver, cortical brain, epididymal fat pad, kidney, adrenals, pancreas, duodenum, diaphragm, and gastrocnemius skeletal muscle were excised, and 300 mg (weight wet) of each tissue was minced in 1 ml of SB at 4°C. For the duodenum, the muscosa of the intestinal wall was separated from the muscularis by scraping under visual inspection through a dissecting microscope. In some cases, only lung tissue was excised after perfusion through the right ventricle at a mean pressure of 20 mmHg with left atrial outflow. The lysates were centrifuged at 13,000 *g* for 1 min at 4°C, and the supernatants (tissue extracts) were processed equivalently for preparative SDS-PAGE and immunoblotting as described above. In the lung and fat pad preparations, the centrifugation step did not pellet all of the tissue debris, and a top layer of fatty material was present and had to be carefully aspirated before the soluble material could be removed. In a few preparations, before tissue excision, the vasculature was perfused for 3 min directly with a protease inhibitor cocktail containing 400 μg/ml benzamidine, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 65 μg/ml aprotinin, 10 μg/ml chymostatin, 100 μg/ml *O*-phenanthroline, and 350 μg/ml phenylmethylsulfonyl fluoride. The presence of protease inhibitors did not alter the observed results.

Immunoblotting of gp60 after interaction with immobilized albumin. A 1% Triton X-100-soluble fraction from the RFC cells was precipitated overnight in 90% ethanol at −20°C. The

pellet was washed once using 70% ethanol and then resuspended in PBS. The suspension was centrifuged at 13,000 *g* for 5 min, and the supernatant was used as the cell extract. Strips of Immobilon or nitrocellulose filters (1 × 0.25 in.) were incubated for 4 h at room temperature in *1*) PBS alone, *2*) 10 mg/ml of albumin in PBS, or *3*) 10 mg/ml of transferrin in PBS. After air drying for at least 1 h, all strips were quenched for 1 h with blocking solution (5% Blotto and 0.1% Nonidet P-40 in PBS) and then incubated overnight at room temperature in 0.5 ml of a 1:50 dilution of the RFC cell extract in blocking solution. Some of the albumin strips and transferrin strips were also incubated in blocking solution without cell extract as additional controls. All strips were washed three times for 5 min with 0.1% Nonidet P-40 in PBS and then cut into small pieces. Proteins were eluted using 100 ml of SB in a boiling water bath for 15 min, separated by SDS-PAGE on a mini-gel apparatus (Bio-Rad), and then electrotransferred onto Immobilon filters at 40 V for 1.5 h. The filters were immunoblotted with α-gp as described above.

RESULTS

gp60 interacts with both α-gp and albumin. Recently, we have shown that α-gp does interact with an RCA- and WGA-binding 60-kDa sialoglycoprotein (26) present on the surface of cultured microvascular endothelium derived from RFC. Other work (23) has implicated an RCA- and WGA-binding 60-kDa glycoprotein as an albumin-binding protein on the RFC cell surface. Because these data strongly suggest that α-gp is interacting with the albumin-binding protein gp60, we attempted to inhibit albumin binding to the surface of cultured RFC cell monolayers using an IgG fraction of α-gp isolated with immobilized protein A as in Ref. 26. The cell monolayers were first exposed for 10 min to the IgG fraction of α-gp (up to 100 μg/ml). Then ^{125}I-albumin was added to achieve a final concentration of 2 μg/ml, and the usual binding assay was performed (22, 23). Although this IgG fraction did interact with the 60-kDa protein by immunoblotting of RFC cell lysates (26), it did not affect albumin binding to the RFC cell surface (data not shown), suggesting different binding sites for the antibody and albumin.[1] Therefore it became necessary first to ensure that α-gp is indeed interacting with the 60-kDa albumin-binding glycoprotein gp60 and not just another surface glycoprotein of similar apparent molecular mass. Albumin was adsorbed to filters and then air dried to immobilize the protein to the filters. After blocking the filters, they were exposed to an RFC cell extract containing gp60 and then washed. Proteins were eluted from the strips, separated by SDS-PAGE, electrotransferred to filters, and then immunoblotted with α-gp. Controls included *1*) exposing the cell extract to filters alone or to transferrin immobilized to filters and *2*) testing the material desorbed from the transferrin and albumin filters that had not been exposed to the cell extract. Figure 1 shows that α-gp detected a single 60-kDa protein only in eluates of the albumin-adsorbed strips interacted with the RFC cell extract. The eluates from all of the control strips were negative. These results indicate that *1*) gp60 interacts preferentially with albumin, *2*) α-gp does indeed recognize gp60 present in the RFC cell extracts, and *3*) α-gp can now be used with confidence as a probe for the albumin-binding glycoprotein gp60.

Endothelial expression of gp60: immunoprecipitation with α-gp of endothelial glycoproteins radiolabeled biosynthetically in culture. Although it is clear that gp60 is located on the surface of vascular endothelium in culture (23, 25), the biosynthetic origin of gp60 is uncertain. Figure 2 shows that gp60 can be immunoprecipitated specifically with α-gp from lysates of RFC cells that had been biosynthetically radiolabeled with tritiated sugars. gp60 was radiolabeled successfully with a mixture of [6-^{3}H]galactose and [6-^{3}H]glucosamine but not with [2,6-^{3}H]mannose alone. To ensure that gp60 was indeed expressed during each radiolabeling procedure, both radiolabeled cell lysates were subjected to SDS-PAGE, and the separated proteins were electrotransferred onto filters that were immunoblotted with α-gp. An equivalent signal for gp60 was detected in both cases (data not shown). These results indicate that gp60 is indeed ex-

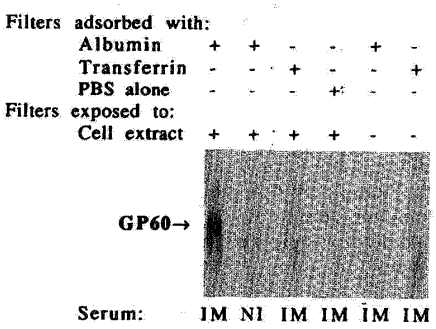

Fig. 1. Specific interaction of 60-kDa glycoprotein (gp60) with albumin immobilized on filters and subsequent detection by immunoblotting with antiserum against glycoprotein (α-gp). Filters were incubated with albumin in phosphate-buffered saline (PBS), transferrin in PBS, or PBS alone (as indicated for each lane above) and then air dried to immobilize the proteins onto filters. As indicated, strips were then either exposed or not exposed to rat epidymal fat pad (RFC) cell extract in a blocking solution. Proteins were eluted from each strip, separated by SDS-PAGE, and electrotransferred onto Immobilon filters. Strips of these filters containing eluted proteins are shown above and were immunoblotted either with nonimmune (NI) and/or immune α-gp (IM) serum. Bound immunoglobulin G (IgG) was detected using anti-rabbit IgG antibodies conjugated to alkaline phosphatase as described previously (25). Gp60 was detected only after interaction of cell extract with albumin filters and not in various controls.

[1] The observation that α-gp, even at high concentrations, does not interfere with albumin binding to the cell surface suggests that albumin and α-gp may interact with gp60 at different binding sites within the molecule. This postulation is supported by our recent investigation attempting to define the epitope recognized by α-gp that indicates that α-gp reacts with a peptide region located apparently in the endodomain of the protein (unpublished observations). Conversely, albumin binding to the endothelial cell surface is expected to be via the ectodomain of gp60. An alternate explanation is supported by our immunofluorescence studies on permeabilized and nonpermeabilized RFC cells, which showed only mild labeling of the cell surface with α-gp (data not shown). Because the antibody was raised against an antigen denatured by SDS-PAGE, it is not surprising that α-gp apparently interacts poorly with the native form of gp60 seen on the cell surface. The epitope may be masked in its native state and/or require some degree of gp60 denaturation before antibody recognition. Furthermore, since our results indicate that the epitope lies in the endodomain of gp60, it may, like glycophorin, interact with cytoskeletal proteins; this interaction may interfere with antibody binding to the permeabilized cells. Unfortunately, the poor immunofluorescence staining with α-gp precludes more exact immunolocalization studies at the electron microscopic level on either cultured cells or tissue. These immunolocalization studies must be performed with a new antibody that recognizes gp60 under more native conditions.

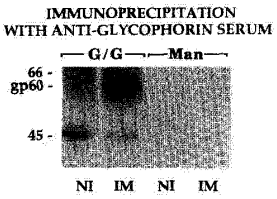

Fig. 2. Biosynthetically radiolabeled glycoproteins of RFC cells immunoprecipitated with α-gp serum. RFC cells were radiolabeled with either [^{3}H]mannose (Man) or both [^{3}H]galactose and [^{3}H]glucosamine (G/G) as described in METHODS. Lysates from these cells were subjected to immunoprecipitation with either nonimmune (NI) or immune (IM) α-gp rabbit serum. Immunoprecipitates were separated by SDS-PAGE on a 5–15% gradient gel and visualized by fluorography. Only gp60 was detected specifically with α-gp. The band found at 45 kDa is considered to be nonspecific since it was detected both in nonimmune serum and to a lesser extent in immune serum.

pressed by vascular endothelium in culture. Furthermore, the lack of incorporation of [2,6-^{3}H]mannose into gp60 is consistent with our previous findings based on direct and sequential lectin affinity chromatography (25) that suggest that rat gp60 contains *O*-linked glycans but apparently not *N*-linked glycans.

Endothelial cell isolation from rat tissues. Fluorescence-activated cell sorting was used to isolate microvascular endothelial cells from a mixture of cells derived from a collagenase treatment of cortical brain, heart, and lung tissues (see METHODS). The specific ability of endothelial cells to internalize DiI-AcLDL was used to isolate endothelial cells from nonendothelial cells (22, 30). Those cells that lacked uptake of DiI-AcLDL were also saved from the heart and lung preparations. After sorting, all cells were replated, grown in culture for several passages, and checked periodically for DiI-AcLDL uptake using either the fluorescence-activated cell sorter or simple fluoresence microscopy of the cells grown on slides as described in Ref. 22. Figure 3 shows the fluorescence profile of DiI-AcLDL uptake of a typical cell preparation. The cells initially isolated from tissue have a bimodal cell distribution with considerable variation in cell size and fluorescence intensity. Most of the cells have fluorescence intensity far greater than the MDCK cells (negative control) and appear to internalize DiI-AcLDL. A second group of cells has a fluorescence intensity more comparable to the MDCK cells, especially when cell size is considered. The top 20% of the cells with the greatest fluorescence intensity and the bottom 20% of the cells with the lowest fluorescence intensity were collected separately using the cell sorter. These cells after growth in culture were examined for DiI-AcLDL uptake once again and, as shown in Fig. 3*B*, the endothelial cells that were positive on the first sort are now unimodal in distribution with considerably greater fluorescence intensity than the negative control. Meanwhile, the other isolated cells shown in Fig. 3*C*, which should be nonendothelial in origin, also appeared to have a unimodal cell distribution; however, in this case, their fluorescence was equivalent to the negative control. Many of the endothelial cell preparations were also checked periodically by immunofluorescence as described in Ref. 22 and in each case stained positively for other endothelial markers, such as angiotensin-converting enzyme or factor VIII (data not shown). This approach has allowed us to isolate

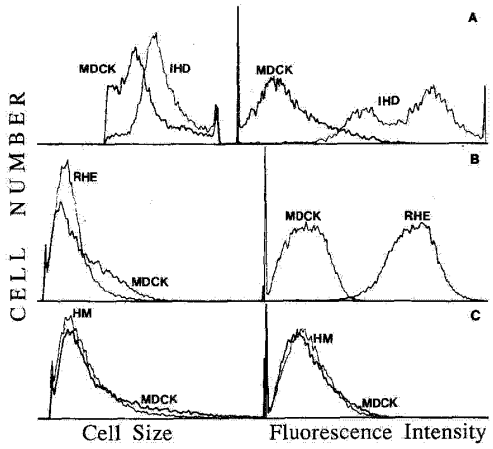

Fig. 3. Profile of fluorescence-activated cell sort of cultured cells derived from a rat heart preparation. Cells were isolated from 4 rat hearts and grown in culture until confluency (see METHODS). These cells were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (DiI-AcLDL) and processed for fluorescence-activated cell sorting as described previously (20, 30). Cell size and fluorescence intensity profiles of cells are shown as a function of cell number. Profiles of initial population of heart-derived (IHD) cells are given in *A*. MDCK cells were also examined as a negative control. Twenty percent of cells with highest and lowest fluorescence intensity were isolated separately using cell sorter and then grown in culture. After 3 passages, these 2 cell populations were reexamined for DiI-AcLDL uptake, and their profiles are shown in *B* [rat heart endothelial (RHE) cells are derived from those cells with highest fluorescence intensity of DiI-AcLDL-positive cells] and *C* [heart mixture (HM) of cells grown from bottom 20% of cells with lowest flourescence]. When total fluorescence of RHE cells was compared with that of MDCK cells, >98% of RHE cells internalized more DiI-AcLDL than any control cells. On the other hand, HM cells exhibited same degree of fluorescence as MDCK cells. [To get a population of cells that lacked DiI-AcLDL uptake (i.e., were devoid of endothelial cells), we found it necessary to passage cells in culture at least twice.]

and grow in culture endothelial cells from the rat heart, lung, and brain and nonendothelial cells from the heart and lung.

Immunoblotting lysates of cultured cells. The proteins from the organ-derived cells isolated and grown in culture (as described above) were solubilized, separated by SDS-PAGE, electrotransferred to filters, and then immunoblotted with α-gp and nonimmune serum. Proteins from RFC cells acted as a positive control. Figure 4 shows that gp60 was present only in the lysates of endothelial cells isolated from the heart and lung but not from the cortical brain.[2] For the heart and lung preparations, only the DiI-AcLDL-positive endothelial cells expressed gp60, whereas the mixture of nonendothelial cells (probably consisting of fibroblasts, pericytes, and/or smooth muscle cells) neither internalized DiI-AcLDL nor appeared to express gp60. These results indicate that under the conditions used here to isolate and grow these cells, microvascular endothelia from the heart and lung but not the brain express gp60 in culture, whereas nonendothelial cells from the heart and lung do not. We have also checked by immunoblotting several specific

[2] gp60 can also be immunoblotted specifically with α-gp using lysates from other cultured endothelium derived from sheep pulmonary artery, bovine aorta, fetal bovine heart (ATCC), microvessels of pig atrium, and human umbilical vein (unpublished observations).

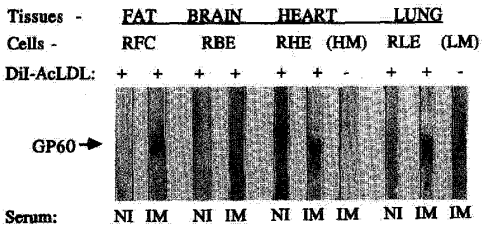

Fig. 4. Differential expression of gp60 in rat microvascular endothelial cells isolated from different tissues. RFC cells along with microvascular endothelial cells isolated from rat brain (RBE), heart (RHE), and lung (RLE) were processed for immunoblotting overnight with a 1:500 dilution of either nonimmune (NI) or immune (IM) α-gp serum. In addition, a mixture of nonendothelial cells isolated from rat heart (HM) and lung (LM) were also immunoblotted with α-gp. In each case, a lysate equivalent of ~10^6 cells was loaded onto a preparative gel. Both endothelial and nonendothelial cells were passaged 3 times in culture after their initial isolation and separation by fluorescence-activated cell sorting. Heart and lung endothelial cells both internalized DiI-AcLDL and expressed gp60, whereas nonendothelial cells did neither. Gp60 was not detected in lysates of cultured brain endothelium.

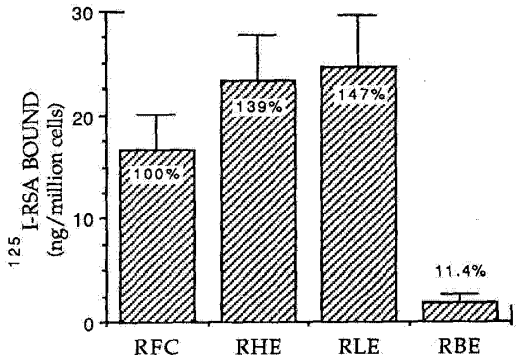

Fig. 5. Differential albumin binding to cultured rat endothelial cells. RFC cells along with microvascular endothelial cells isolated from rat brain (RBE), heart (RHE), and lung (RLE) were examined for their ability to bind radioiodinated rat serum albumin (RSA) using 0.1 mg/ml of ^{125}I-RSA in an in vitro assay described previously (20). For each endothelial cell type, mean value of binding observed from 4 cell monolayers in dishes is expressed as ng/10^6 cells, with error bars denoting calculated SD values. Data were also normalized relative to RFC cells and are expressed as a percentage, which is written above or within columns representing each cell type.

nonendothelial cell types grown in culture. Normal rat kidney fibroblasts [from American Type Culture Collection (ATCC); NRK-49F] that bind albumin poorly (22) and rat aortic smooth muscle cells (from ATCC; A10) were negative for gp60 expression (data not shown). Therefore, in the cultured cells tested so far, the expression of gp60 appears to be specific for certain endothelial cells.

Selective albumin binding to rat cultured endothelial cells. Because the data given above indicate that gp60 expression is limited to only certain endothelial cells [rat heart endothelial (RHE), RFC, and rat lung endothelial (RLE) but not rat brain endothelial (RBE) cells], the binding of albumin to these cells should vary in accordance with gp60 expression if gp60 does indeed play a role in albumin binding. Rat serum albumin (RSA) was radioiodinated, and the binding of ^{125}I-RSA to the surface of these cultured cells was compared using 0.1 mg/ml of ^{125}I-RSA as described previously (22). Because the RFC cells bind RSA and were used originally to characterize the kinetics of albumin binding to cultured microvascular endothelium (22), they acted as a positive control. As shown in Fig. 5, ^{125}I-RSA binding was ~10–15 times greater for the RLE, RHE, and RFC cells than for the RBE cells. This small amount of binding of albumin to the RBE cells is comparable to that observed previously for rat fibroblasts (22). This selective albumin binding to certain cultured endothelial cells agrees well with the above observation that the RLE, RHE, and RFC cells expressed gp60 but the RBE cells did not. In addition, the increase of 40–50% in ^{125}I-RSA binding observed for the RLE and RHE cells over the RFC cells correlates well with the greater expression of gp60 by these cells (see Fig. 4).

Presence of gp60 on the surface of microvascular endothelium in situ. Most of our previous work has focused on using in vitro systems to study molecular interactions at the surface of the vascular endothelium. Recently, we have examined the extent of phenotypic drift caused by cell culture by performing intravascular radioiodinations of endothelial surface proteins to compare those proteins identified in situ with those in culture (25). Here we

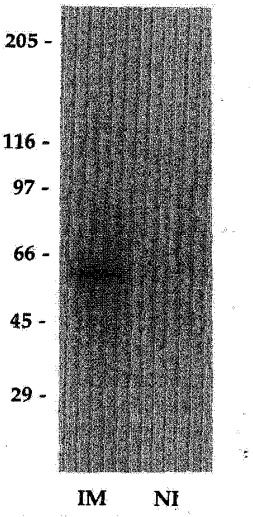

Fig. 6. Intravascular radioiodinated surface proteins of heart vascular endothelium immunoprecipitated with α-gp serum, separated by SDS-PAGE, and visualized by autoradiography. Heart extracts with ^{125}I-labeled endothelial luminal surface proteins (24) were subjected to immunoprecipitation with either nonimmune (NI) or immune (IM) α-gp serum. Because it appears that this type of radioiodination in situ is limited to luminal surface of endothelium (25), these results indicate that α-gp recognizes intravascularly radioiodinated gp60 that is located on luminal surface of heart microvascular endothelium in situ. Molecular mass standards used were myosin (205 kDa), β-galactosidase (117 kDa), phosphorylase B (97 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa).

show that gp60 is indeed a molecular component of the endothelial glycocalyx in situ by using α-gp to immunoprecipitate radiolabeled gp60 from heart extracts containing microvascular endothelial proteins that have been radioiodinated in situ. As described previously (25), the luminal surface proteins of the myocardial microvascular endothelium were radioiodinated by intravascular perfusion of Na^{125}I and small microspheres conjugated with both lactoperoxidase and glucose oxidase. As shown

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