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Albondin-mediated Capillary Permeability to Albumin

DIFFERENTIAL ROLE OF RECEPTORS IN ENDOTHELIAL TRANSCYTOSIS AND ENDOCYTOSIS OF NATIVE AND MODIFIED ALBUMINS*

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Specific albumin binding to the surface of endothelium initiates its transcytosis across continuous endothelium via noncoated plasmalemmal vesicles. Past work has identified several putative albumin-binding proteins (SPARC, gp60, gp30, and gp18). In this study, we examined the specific role of these proteins in the binding of bovine serum albumin (BSA) to endothelium. The 60-kDa albumin-binding protein (gp60, now called albondin) was purified from cultured rat microvascular endothelial cells for antibody production. Anti-albondin antibodies (αgp60) specifically recognized albondin expressed by rat, bovine, and human endothelial cells (EC). αgp60 and unlabeled native BSA inhibited ^{125}I-BSA binding to confluent EC monolayers and to both albondin and SPARC extracts immobilized on filters. Modification of BSA by maleic anhydride treatment (Mal-BSA) or by surface adsorption to colloidal gold particles (A-Au) renders the ligand specific for gp30 and gp18 while eliminating its ability to inhibit ^{125}I-BSA binding to EC and to both albondin and SPARC extracts. Mal-BSA and A-Au interacted with EC via distinct binding sites not recognizing native BSA. EC internalization of ^{125}I-BSA is inhibited by unlabeled BSA and αgp60 IgG but not nonimmune IgG, Mal-BSA, or A-Au. Conversely, internalization of modified BSA is inhibited by unlabeled modified BSA but not BSA or αgp60 IgG. Specific ^{125}I-BSA transendothelial transport in rat lungs perfused *in situ* and for EC monolayers *in vitro* is inhibited (≥90%) by unlabeled BSA and αgp60 IgG but not nonimmune IgG and Mal-BSA. No specific transport of ^{125}I-Mal-BSA is detected across bovine lung microvascular endothelial cell monolayers, only paracellular and/or fluid-phase transport. Low temperatures reduce BSA transport by 5-fold and Mal-BSA by 2-fold. Interestingly, 3-fold more native BSA is transported than Mal-BSA at 37 °C, whereas at 8–10 °C they are nearly equal, suggesting facilitation of BSA transport. Cumulatively, it appears that gp30 and gp18 mediate the binding, endocytosis, and degradation of modified albumins, whereas albondin mediates native albumin binding which significantly enhances its transcytosis and capillary permeability.

The endothelium lining many microvascular beds of many organs is the significant barrier to the transvascular transport of plasma proteins and metabolites from the capillary vessels to the interstitial fluid. Serum proteins circulating within the vasculature may interact with the endothelial cell surface either to enhance or to hinder transcapillary exchange. Such interactions appear to be required to establish normal capillary permeability (1–7). In a phenomenon called the "serum effect," the interaction of circulating proteins, such as albumin and orosomucoid, with the endothelial cell surface of many microvascular beds (5, 8–14) significantly restricts transcapillary transport of a wide spectrum of molecules (1–7). Conversely, specific binding mechanisms may exist to enhance the transendothelial exchange of a selective group of ligands in order to provide specific nutrients to tissue cells in accordance with their particular needs. Endothelial surface receptors or transporters for plasma molecules including transferrin (15, 16), insulin (17), orosomucoid (8), ceruloplasmin (18), lipoproteins (19), and even glucose (20) have been detected in select tissues and may mediate specific transport into and/or across the endothelium. A few direct anti-endothelial cell receptor conjugates have been created for targeting drugs to specific organs (21). Vectorial receptor-mediated transport may also occur in the opposite direction. Lipoprotein lipase synthesized by underlying adipocytes and myocytes is transported by a saturable mechanism across the endothelium to the luminal surface where it stays bound and active (22).

The serum protein albumin is a multifunctional carrier protein that contributes to cardiovascular fluid homeostasis through osmotic forces and permselectivity ("serum") effects (2–6) and to the transport, distribution and metabolism of a wide variety of small blood-borne molecules including fatty acids, sterols, amino acids, hormones, metal ions, and numerous drugs (23, 24). It has a dual role in the permeability of many microvascular beds lined with a continuous type of endothelium. As a major contributor to the serum effect, albumin binds the surface of endothelium (5, 9–14) and reduces the transvascular flux of water, solutes, and macromolecules (2–6). Albumin binding to the endothelial glycocalyx creates a more restrictive endothelial barrier, possibly via cell membrane-transduced signals that modulate intracellular calcium levels (25) and/or the formation of an additional permselective barrier within transendothelial transport pathways (5, 6, 13, 14, 26). Conversely, albumin can enhance specific transport by acting as a carrier protein in the circulation for its many small ligands (23, 24). Albumin is actively involved in the presentation of important ligands such as fatty acids to a variety of tissue cells including adipocytes for the storage of fat (27), Leydig cells for hormonal production (28), and myocytes for energy production via oxidative metabolism (29). Because the endothelial barrier can prevent macromolecular transport from the circulating blood to the extravascular space, it is unclear whether certain

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endothelia play a direct role in selective albumin transport. However, it is known that (i) many continuous endothelia have specific albumin binding sites apparently for transcytosis via an abundant population of noncoated plasmalemma vesicles (9, 11–13, 30); (ii) ligands bound to albumin such as fatty acids appear to increase its endothelial binding and transcytosis (24); and (iii) cultured endothelium can internalize and release albumin with little degradation (31, 32). Native albumin transport across continuous endothelium via noncoated plasmalemmal vesicles or caveolae (11, 12, 30) provides a carrier mechanism for the facilitated delivery of its small ligands from the circulating blood to the interstitium in a manner consistent with the nutritional requirements of the underlying tissue cells.

Albumin specifically binds the endothelial cell surface in a manner consistent with a receptor-ligand interaction (13, 14). Various putative albumin-binding proteins have been suggested over the past decade (31–38). Two of these proteins, gp30 and gp18, were recently shown to recognize modified albumins much more avidly than native albumin (31) and appear to mediate the high affinity binding of modified albumins to the cell surface, ultimately for endocytosis and lysosomal degradation (31, 32). Two other albumin-binding proteins, gp60 (14, 34) and SPARC (33), are functionally and immunologically related, possibly via a common albumin binding domain (37).

In this study, we show that direct binding of native albumin to the 60-kDa endothelial cell surface protein, gp60, now renamed albondin, can mediate its transendothelial transport. Specific antibodies raised against purified albondin inhibit not only albumin binding to the endothelial cell surface but also capillary permeability to albumin by reducing its internalization and transport across the endothelium. In addition, we show that modification of albumin can prevent its albondin-facilitated transport and that native and modified albumins interact distinctly via different binding sites on the cell surface for differential cellular internalization and processing.

EXPERIMENTAL PROCEDURES

Materials—Reagents and other supplies were obtained from the following sources. Fetal calf serum and phosphate-buffered saline (PBS)[1] were from Life Technologies, Inc. Gelatin was from Difco.Crystallized bovine serum albumin (BSA) was from ICN Biochemicals. *Triticum vulgaris* agglutinin (wheat germ agglutinin) conjugated to agarose beads, BSA-agarose beads, *N*-acetylglucosamine, bovine transferrin, and orosomucoid was from Sigma. Dulbecco's modified Eagle's medium (DMEM) was from Irvine Scientific. IODO-GEN (1,4,5,6-tetrachloro-3α,6α-diphenylglycouril), Triton X-100, SDS, and the bicinchoninic acid protein assay were from Pierce Chemical Co. $Na^{125}I$ was from Amersham Corp. All tissue culture plasticware was from Costar (Cambridge, MA) or Corning (Wilmington, DE). Transwell porous cell culture inserts (24 mm, 0.4-μm pore, Nucleopore polycarbonate membrane, tissue culture-treated) were from Costar. [^{14}C]Inulin was from ICN. Ferritin was from Polysciences, Inc. (Warrington, PA). Nitrocellulose filter was from Schleicher & Schuell, and Immobilon was from Millipore (Bedford, MA).

Cell Culture—Microvascular cells, derived from rat epididymal fat pads (RFC) and bovine lungs (BLMVEC), along with bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial cells (HUVEC) were obtained, grown in culture, and tested periodically for endothelial markers as in our past work (8, 13, 31, 37, 38).

Molecular Probes—BSA was modified with maleic anhydride (Mal-BSA) and by surface adsorption to colloidal gold particles (A-Au) as described (31, 32). BSA, ferritin, and Mal-BSA were radioiodinated using IODO-GEN as in Ref. 8. ^{125}I-A-Au was created by using a 1:100 ratio of radiolabeled to unlabeled BSA as in Refs. 31 and 32.

BSA Cell Binding Assays—Our past ligand binding assay (8, 13) was performed using BLMVEC grown to confluence in six-well trays. Briefly, after washing the cell monolayers extensively at 37 and then 4 °C, they were incubated at 4 °C first with the potential inhibitor (or none) in DMEM for 10 min and then with ^{125}I-BSA (0.1–0.3 μg/ml) in the same solution. After washing with DMEM at 4 °C, the monolayers were solubilized and the lysates counted for radioactivity using a Beckman 5500B γ-counter.

Antiserum Production—Albondin extract (100 μg) was used with complete Freund's adjuvant to inoculate a New Zealand White rabbit. Subsequent boosting injections used incomplete Freund's adjuvant with 50 μg of antigen that was electroeluted from the 60-kDa band of a SDS-polyacrylamide gel. To be certain that this antiserum does not interact with albumin which can copurify with albondin before wheat germ agglutinin affinity chromatography (as assessed by enzyme-linked immunosorbent assay and Western blotting), we routinely run this antiserum over a BSA-agarose column. The flow-through is labeled αgp60, is used for all experiments described herein, and does not recognize BSA by enzyme-linked immunosorbent assay and Western blotting. We also tested for possible interactions in solution of αgp60 IgG (50 μg/ml) with ^{125}I-BSA (0.2 μg/ml) by gel chromatography using a G-200 column and found that the antibody did not cause any ^{125}I-BSA to elute earlier at a higher molecular weight position whereas anti-BSA antibody did, indicating a lack of αgp60 interaction with BSA in solution.

Inhibition of ^{125}I-BSA Binding to Immobilized Albondin and SPARC—Direct ^{125}I-BSA binding assays were performed on extracts of albondin and SPARC immobilized on nitrocellulose filters. gp60 extract (0.25–0.5 μg) or SPARC (0.25–0.5 μg) isolated from BAEC medium as in Ref. 33 or purchased from Haematologic Technologies Inc. (Essex Junction, VT; human form) was blotted onto nitrocellulose and air dried. The dot blots were blocked for 1 h at 4 °C with PBS containing 5% Blotto and 0.5% Triton X-100 and preincubated for 2 h with the potential inhibitor in blocking solution. Then, ^{125}I-BSA was added to achieve a final concentration of 0.2 μg/ml; after 10 min, the filters were washed (three times for 1 min each) with PBS containing 0.5% Triton X-100 and then counted for radioactivity.

Inhibition of BLMVEC Uptake of ^{125}I-labeled Native and Modified BSA—BLMVEC monolayers were washed extensively, preincubated for 10 min at 37 °C first with DMEM in the presence of the potential inhibitors (IgGs at 100 μg/ml and albumins at 1 mg/ml); then ^{125}I-labeled BSA, A-Au, or Mal-BSA was added to a final concentration of 0.2 μg/ml. After 20 min, the medium was removed, and the cells were washed with DMEM at 4 °C (three times for 1 min each) and then subjected as described previously (32) to Pronase digestion to determine ligand specifically internalized by the cells.

Permeability Measurements in Rat Lung in Situ—The "single sample" injection method (39, 40) was used to assess the permeability-surface area product (*PS*) of albumin in rat lung in the presence of various potential inhibitors of its transport. The lungs of anesthetized male Sprague-Dawley rats were ventilated and then perfused via the pulmonary artery as described previously (14). Briefly, the right ventricle was injected with 0.5 ml of Ringer's solution at pH = 7.4 (111 mM NaCl, 2.4 mM KCl, 1 mM $MgSO_4$, 5.5 mM glucose, 5 mM HEPES, 0.195 mM $NaHCO_3$, 1.1 mM $CaCl_2$) containing 30 μM nitroprusside and 350 units of heparin before cannulation of the pulmonary artery. The lungs were flushed at 10 mm of Hg with the following solutions: (i) oxygenated Ringer's solution containing 30 μM nitroprusside for 3 min; (ii) oxygenated Ringer's plus blockers/inhibitor(s) for 1.5 min; (iii) the solution in step ii plus trace ^{125}I-BSA (0.3–1.0 μg/ml) or ^{125}I-ferritin (1 μg/ml) for 2 min; and (iv) the solution in step i for 2 min. The lungs were excised, groomed, and cut into 14 samples. Each sample was weighed and counted for radioactivity. Based on previous work (12, 39), the chosen perfusion times not only provide albumin enough time to cross the endothelium, which is rapidly transcytosed after only 15–30 s, but also minimize with vascular flushing the presence of albumin within the cell or at the cell surface. In this way, the detected radioactivity will gage tissue clearance across the endothelial barrier and will predominantly represent ^{125}I-BSA that has been transcytosed and released to the interstitium. Also, note that the flush time is too short to allow any loss of ^{125}I-BSA to lymphatic drainage and that the perfusion pressures and flow rates were constant with antibody and albumin perfusions, indicating little, if any, variation in the vascular surface area.

The *PS* was calculated as experimentally and theoretically defined in (39) using

$$PS = A/t \times C_p$$

[1] The abbreviations used are: PBS, phosphate-buffered saline; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; RFC, rat fat pad capillary; BAEC, bovine aortic endothelial cell(s); BLMVEC, bovine lung microvascular endothelial cell(s); HUVEC, human umbilical vein endothelial cell(s); Mal, maleic anhydride; A-Au, albumin adsorption to gold particles (albumin-gold complexes); PAGE, polyacrylamide gel electrophoresis; *PS*, permeability-surface area product; NI, nonimmune serum; LDL, low density lipoprotein; αgp60, anti-albondin serum; BON, anti-SPARC serum; PI, preimmune serum.

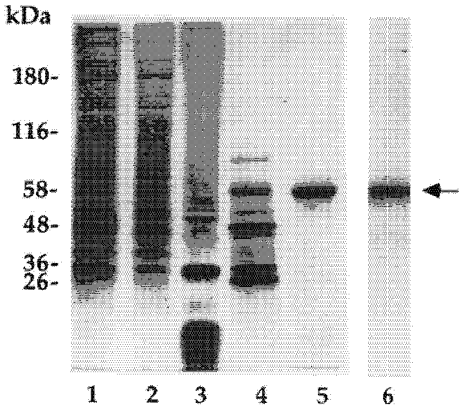

FIG. 1. **Purification of albondin.** SDS-PAGE analysis of fractionation steps used to purify albondin (silver stain of equivalent protein loads). *Lane 1*, total cell lysate. *Lanes 2–6*, proteins of various fractions of purification procedure. RFC proteins were extracted using Triton X-100 (*lane 2*) followed by ethanol precipitation as described in Ref. 6. The precipitate was resuspended in PBS, mixed for 1 h, and then centrifuged at 4 °C. The proteins in the pellet and supernatant are shown in *lanes 3* and *4*, respectively. After the addition of detergent to a final concentration of 1% Triton X-100 and 0.2% SDS, the supernatant was subjected to wheat germ agglutinin affinity chromatography followed by elution with 0.5 M *N*-acetylglucosamine. The eluant was dialyzed against 0.1 N PBS containing 0.1% Triton X-100 and 0.02% SDS and then concentrated 3-fold by Speed-Vac. SDS-PAGE analysis of this last fraction revealed only one band at 60 kDa by silver staining (*lane 5*) and after radioiodination (*lane 6*) as in Refs. 8 and 14.

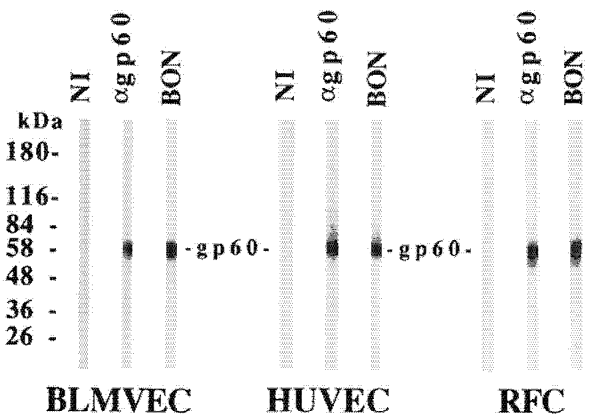

FIG. 2. **Antisera recognition of albondin in endothelial cell lysates by immunoblotting.** As described previously (32, 42), the proteins of cultured monolayers from BLMVEC, RFC, and HUVEC were solubilized and analyzed by SDS-PAGE followed by electrotransfer to Immobilon filters for immunoblotting with the indicated sera. As a positive control and in agreement with our past work (37), antiserum BON made against the secreted 43-kDa albumin-binding protein, SPARC, also recognizes gp60.

where A = cpm/g of wet lung, t = perfusion time with ^{125}I-BSA = 2 min, and C_p= cpm/ml of ^{125}I-BSA perfusate. Because recent work (25) suggested that the absence of Ca^{2+} from perfusates may prevent the normally large increase in hydraulic conductivities found with removal of albumin from perfusates, we also performed many experiments using Ringer's solution without Ca^{2+}. We did not observe any significant differences in BSA transport in the presence or absence of Ca^{2+}. Therefore, all experiments were combined.

In Vitro Transport Assay—BLMVEC (12,000 cells/well) were seeded onto Transwell filters (0.33 cm^2). After 6–7 days, transmonolayer transport assays were performed similar to previous work (41). This filter system separates a well of a 24-well plate into a top and bottom chamber by a polycarbonate filter (0.4-μm diameter pores) covered by confluent BLMVEC. Volumes of 100 μl for the top chamber and 600 μl for the bottom chamber were used to create an equal fluid line in each chamber and minimize hydrostatic effects. The cells were washed at 37 °C with Ringer's solution (twice for 1 min each), and then Ringer's solution containing 0.5 mg/ml bovine orosomucoid (once for 10 min, once for 1 min). Ringer's solution containing orosomucoid was added above to the bottom chamber, and with the desired potential inhibitor to the top chamber. After 15 min, the fluid in the top chamber was replaced with Ringer's solution containing orosomucoid containing ^{125}I-BSA (0.2 μg/ml) and the same potential inhibitor. To compare transport across the monolayers, we removed 30 μl of fluid from the bottom after 30 min. The samples were counted for γ radioactivity.

RESULTS

Purification of Albondin—We have purified the 60-kDa endothelial protein, gp60 (now called albondin), for which our initial evidence suggested a role in albumin binding (14, 30). Albondin was initially extracted from RFC cell monolayers using Triton X-100 followed by ethanol precipitation as described in Ref. 14. The precipitate was resuspended in PBS containing 1% Triton X-100 and 0.2% SDS and was subjected to wheat germ agglutinin affinity chromatography followed by elution with 0.5 M *N*-acetylglucosamine. Fig. 1 shows that SDS-PAGE analysis of this eluted fraction revealed only one band at 60 kDa by silver staining (also true for Coomassie staining; data not shown) and after radioiodination of this fraction. Densitometry revealed that greater than 98% of the detected signal was present in the single band. Because our past experiments showed that antiserum (BON) made against the secreted 43-kDa albumin-binding protein, SPARC recognized albondin (37), we used BON in slot blots and Western blots to assay each of our fractions for the presence of albondin. We also used ^{125}I-BSA on slot blots for following albumin binding activity. Our average yield in the final fraction for three different isolations was 47% with a 710-fold degree of purification. To be certain that the purified 60-kDa protein is indeed responsible for the albumin binding activity, we incubated an aliquot of the final fraction with BSA conjugated to agarose beads and found that these beads extracted almost all of the 60-kDa protein from the solution, whereas equivalent IgG beads did not.

Specific Recognition of Albondin by Antiserum—Polyclonal rabbit antiserum (αgp60) was made against albondin extract. Fig. 2 shows that αgp60 specifically recognizes a 60-kDa protein in human, bovine, and rat endothelial cell lysates by immunoblotting. Fig. 3 shows that αgp60 also immunoprecipitated a radiolabeled 60-kDa protein from lysates of endothelial cells whose surface proteins have been radioiodinated. Nonimmune serum (NI) was nonreactive, whereas BON recognized albondin as in our past work (37). Both BON and αgp60 recognized purified albondin by immunoblotting (data not shown). Please note that (i) the 43-kDa protein, SPARC, was not detected in the endothelial cell lysates by BON or αgp60 (consistent with its expression only during cell migration and growth and cessation of expression with cell monolayer confluence (33, 37)); (ii) SPARC was not present on the cell surface as assessed by radioiodination followed by immunoprecipitation (light band near 46 kDa in Fig. 3 is present in all three lanes); and (iii) neither BON nor αgp60 recognized the smaller putative albumin-binding proteins, gp30 and gp18, whose presence on the filters was verified by blotting with A-Au as in Ref. 31.

αgp60 Recognizes SPARC—Since BON interacts with albondin, we tested the ability of αgp60 to recognize SPARC. Fig. 4 shows that αgp60 and BON immunoprecipitated SPARC that was biosynthetically radiolabeled *in vitro*, whereas NI did not. BON and αgp60 also can immunoblot SPARC (data not shown). It appears that these two albumin-binding proteins contain common immunological epitopes.

Antibodies Recognizing Albondin Inhibit ^{125}I-BSA Binding to Endothelium—Next, the IgG fractions from various antisera were used to attempt to inhibit the binding of ^{125}I-BSA to the

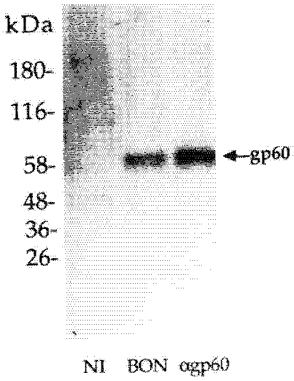

FIG. 3. **Immunoprecipitation of surface radiolabeled albondin from BLMVEC lysates.** Confluent BLMVEC monolayers were radioiodinated using lactoperoxidase, $Na^{125}I$, and hydrogen peroxide and then processed for immunoprecipitation using the indicated antisera followed by SDS-PAGE and autoradiography as described previously (48). Similar results were found using RFC monolayers.

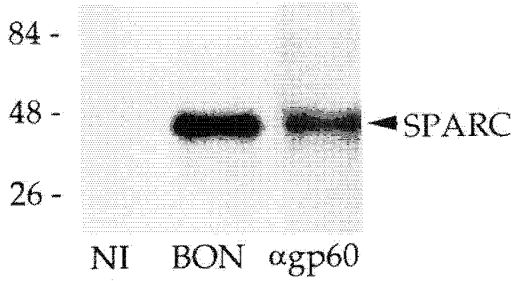

FIG. 4. **Recognition of SPARC by αgp60.** SPARC was biosynthetically radiolabeled with [^{35}S]methionine and immunoprecipitated with the indicated antisera from the culture medium of BAEC as in Ref. 37.

surface of confluent monolayers of cultured BLMVEC.[2] Fig. 5 shows that specific ^{125}I-BSA binding can be ablated almost totally by αgp60 IgG and unlabeled BSA in a concentration-dependent manner. The maximal inhibitory effect of αgp60 (~90%) was comparable to the inhibition attained with a large molar excess of BSA. Fig. 6 shows that BON IgG inhibited this binding by greater than 80%, whereas other antibodies were ineffective. The effects of 20–50 μg/ml of BON and αgp60 IgG were comparable to the inhibition achieved with 1 mg/ml BSA. It appears that specific antibody interaction with albondin significantly reduces cell surface binding of albumin.

Native but Not Modified Albumins Inhibit ^{125}I-BSA Binding to BLMVEC—Endothelium can bind not only native albumins (5, 11–14, 34, 37) but also albumins modified by surface adsorption to colloidal gold particles (A-Au) or by chemical means, such as treatment with maleic anhydride (9, 10, 31, 32, 42, 43). To assess if native and modified albumins interact with the same binding sites, we tested the ability of these albumins to inhibit ^{125}I-BSA binding to the BLMVEC surface. As shown in Fig. 6, unlabeled native BSA inhibited ^{125}I-BSA binding, whereas A-Au and Mal-BSA did not (even at more than 100-fold molar excess and at levels known to saturate gp30 and gp18 binding (31, 32)). Endothelial cells kinetically have two distinct populations of binding sites: one for native albumin and another for modified albumins.

[2] Most of our past work has focused on the RFC cell monolayers (13, 14, 32, 34); however, we found that the RFC and HUVEC monolayers do not form restrictive barriers on filters whereas the BLMVEC do. Furthermore, these BLMVEC monolayers show less signs of phenotypic drift in culture and have a greater density of noncoated plasmalemma vesicles than the other cultured endothelial cells (our unpublished observations).

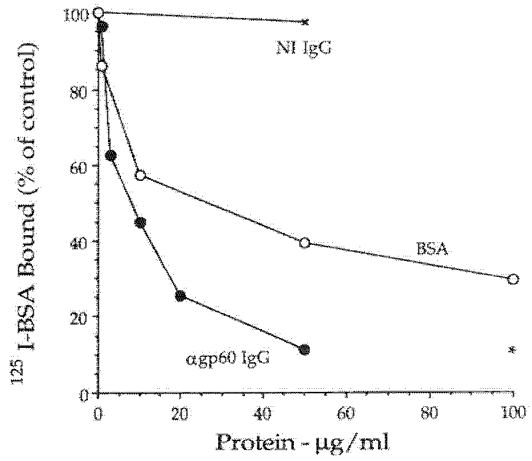

FIG. 5. **Concentration dependence of inhibitors of ^{125}I-BSA surface binding to BLMVEC monolayers *in vitro*.** The indicated proteins (unlabeled BSA, αgp60 IgG, or NI IgG) were tested at different concentrations for their ability to prevent specific ^{125}I-BSA binding to confluent BLMVEC monolayers at 4 °C. In each experiment, duplicate wells were assayed in the presence of a large excess of unlabeled BSA (10–70 mg/ml) to assess the nonspecific background radioactivity. This background was subtracted from all other data points as in Ref. 8 and comprised less than 15% of the total. The final results shown in the graph were normalized to the controls (without inhibitor) and are expressed as a percentage of the control. The mean is given for $2 \leq n \leq 6$. The S.D. were less than 25%. The *asterisk* indicates the detected binding in the presence of 1 mg/ml BSA.

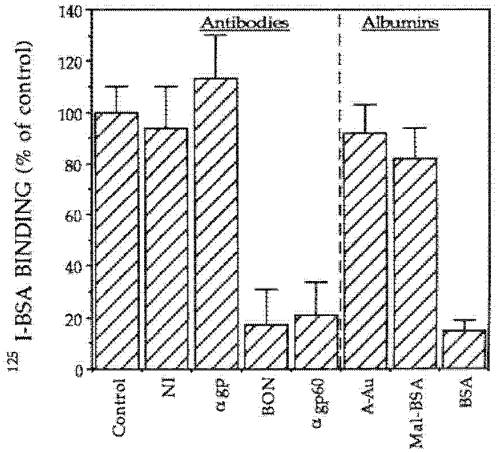

FIG. 6. **Differential inhibition of ^{125}I-BSA binding to the endothelial cell surface.** The IgG fraction of the indicated sera were used (see "Experimental Procedures") at a concentration of 20–50 μg/ml, whereas BSA and Mal-BSA were at 1 mg/ml. The albumin-gold complexes were used at a final concentration of 20–40 μg/ml of BSA conjugated to gold (which saturates binding to gp30 and gp18 (31, 32)). Nonspecific background was assessed and subtracted as described in the legend of Fig. 5. The results are expressed as the percentage of the control filters (no inhibitor). The mean ± S.D. is plotted ($n \geq 4$). The αgp serum has been described previously (14, 32).

Inhibition of Specific ^{125}I-BSA Binding to Immobilized Albondin and SPARC—^{125}I-BSA binding assays were also performed on albondin and SPARC immobilized on nitrocellulose filters (see "Experimental Procedures") in the presence or absence of various antibodies and albumins. As shown in Fig. 7, the IgG fraction of BON and αgp60 but not other antisera

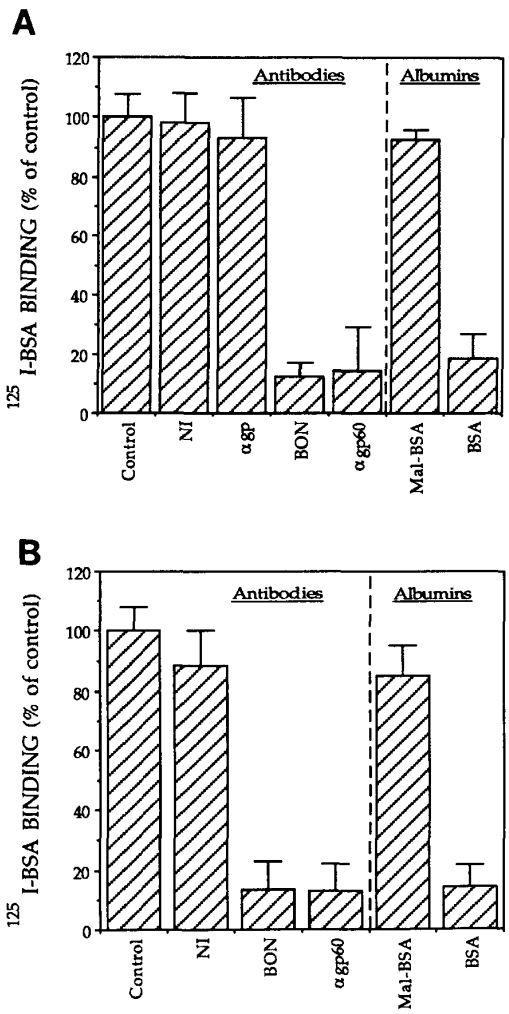

FIG. 7. **Inhibition of ^{125}I-BSA binding directly to albumin-binding proteins on filters.** ^{125}I-BSA binding assays were performed on extracts of gp60 (*panel A*) and SPARC (*panel B*) immobilized on nitrocellulose filters (see "Experimental Procedures") in the presence or absence of the indicated potential inhibitor (concentrations are the same as in Fig. 6). In each experiment, nonspecific background binding was assessed by performing the assay in the presence of excess unlabeled BSA (50–70 mg/ml) and was subtracted from the counts of the other filters. It was less than 25 and 35% of the total counts on the control filters for albondin and SPARC, respectively. The results are expressed as the percentage of the control filters (no inhibitor). The mean ± S.D. is plotted ($n \geq 4$). The results using the human and bovine SPARC were very similar and therefore were combined. Our past work (31) showed poor binding of ^{125}I-BSA to gp30 and gp18 by blotting.

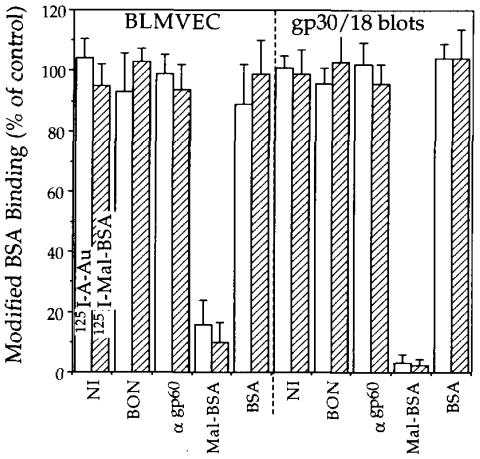

FIG. 8. **Inhibition of modified albumin binding to the surface of BLMVEC and gp30/18 on blots.** *Panel A*, binding inhibition assays (see "Experimental Procedures") were performed on BLMVEC using ^{125}I-A-Au (2 μg/ml) or ^{125}I-Mal-BSA (0.2 μg/ml) with the indicated molecules (50 μg/ml for IgGs and 1 mg/ml for albumins). Nonspecific background detected in the presence of 1 mg/ml unlabeled Mal-BSA was less than 30% of the total without inhibitor and was subtracted from the other monlayers. *Panel B*, inhibition studies were done using blots of gp30 and gp18 as described previously (34). The results for gp30 and gp18 were very similar and therefore were combined. The results represent the total detected binding to gp30 and gp18 expressed as the mean percentage ± S.D. of the control (no inhibitor) for $n \geq 3$.

significantly inhibited binding to both albondin and SPARC. This inhibitory effect was significantly diminished when these IgGs were preincubated with (i) thin filter strips containing immobilized albondin or SPARC processed for preparative SDS-PAGE and then electrotransferred onto Immobilon filters or (ii) immobilized and soluble purified SPARC or gp60 extract, but not (i) immobilized or soluble ovalbumin or (ii) filter strips of a nonspecific region of a preparative SDS gel of BLMVEC lysate transferred onto Immobilon filters (data not shown). Unlabeled native BSA significantly inhibited ^{125}I-BSA binding, whereas Mal-BSA did not. These results correlate well with our findings on native BSA binding to the cell surface and provide evidence that albondin binds native albumin, possibly via a common binding domain shared with SPARC.

Selective Inhibition of ^{125}I-labeled Modified BSA Binding to BLMVEC and gp30/18—We also examined the ability of these probes to prevent the binding of ^{125}I-labeled modified albumins to BLMVEC and to gp30 and gp18 on blots. Fig. 8 shows that native BSA and the antibodies were quite ineffective inhibitors, whereas Mal-BSA inhibited both ^{125}I-A-Au and ^{125}I-Mal-BSA binding very well. Cumulatively, it appears that different proteins mediate the binding of native and modified albumins to the cell surface, with gp30 and gp18 recognizing the modified albumins and albondin interacting with native albumin.

Inhibition of BLMVEC Internalization of Native and Modified Albumins—Having dissected the cell surface binding of these ligands, we proceeded to focus next on their internalization by the cells. Our recent work (31, 32) showed that native and modified albumins are all internalized but are processed differently with only modified albumins being degraded. Herein, we attempted to inhibit the cellular uptake of these ligands by using the same group of potential inhibitors tested in the binding assays. Fig. 9 shows that native BSA and the IgG fraction of BON and αgp60 inhibited specific BLMVEC uptake of ^{125}I-BSA but not ^{125}I-labeled modified albumins. Conversely, Mal-BSA prevented both ^{125}I-Au and ^{125}I-Mal-BSA internalization but not ^{125}I-BSA uptake. NI IgG did not inhibit any ligand uptake. These results show the same inhibition profile as the cell surface binding studies and indicate that native and modified albumins are internalized by different molecular mechanisms. Albondin mediates native albumin internalization, whereas gp30 and gp18 may be responsible for modified albumin uptake.

Selective Inhibition of Capillary Permeability to Albumin in Rat Lungs in Situ—Although the experiments described above provide evidence that albondin is the major endothelial plasmalemma protein mediating the cell surface binding of native albumin, its direct role in the transendothelial transport of albumin is not established. Therefore, we also investigated the

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