Quantitative analysis of albumin uptake and transport in the rat microvessel endothelial monolayer

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Quantitative analysis of albumin uptake and transport in the rat microvessel endothelial monolayer. Am J Physiol Lung Cell Mol Physiol 284: L187–L196, 2003; 10.1152/ajplung.00152.2002.—We determined the concentration dependence of albumin binding, uptake, and transport in confluent monolayers of cultured rat lung microvascular endothelial cells (RLMVEC). Transport of 125I-albumin in RLMVEC monolayers occurred at a rate of 7.2 fmol·min⁻¹·10⁶ cells⁻¹. Albumin transport was inhibited by cell surface depletion of the 60-kDa albumin-binding glycoprotein gp60 and by disruption of caveolae using methyl-β-cyclodextrin. By contrast, gp60 activation (by means of gp60 cross-linking using primary and secondary antibodies) increased 125I-albumin uptake 2.3-fold. At 37°C, 125I-albumin uptake had a half time of 10 min and was competitively inhibited by unlabeled albumin (IC₅₀ = 1 µM). Using a two-site model, we estimated by Scatchard analysis the affinity (K_D) and maximal capacity (B_max) of albumin uptake to be 0.87 µM (K_D1) and 0.47 pmol/10⁶ cells (B_max1) and 93.3 µM (K_D2) and 20.2 pmol/10⁶ cells (B_max2). At 4°C, we also observed two populations of specific binding sites, with high (K_D1 = 13.5 nM, 1% of the total) and low (K_D2 = 1.6 µM) affinity. On the basis of these data, we propose a model in which the two binding affinities represent the clustered and unclustered gp60 forms. The model predicts that fluid phase albumin in caveolae accounts for the bulk of albumin internalized and transported in the endothelial monolayer.

capillary permeability; vesicular transport; caveolae; albumin-binding glycoprotein gp60

THERE IS ACCUMULATING EVIDENCE from morphological and functional studies that albumin is continuously exchanged between the microvascular and interstitial compartments by receptor-mediated albumin transcytosis (8, 11, 13, 16–19, 23, 27–29). Recent studies show that albumin molecules can associate with surface glycoproteins such as gp60 (albondin) in endothelial cells, which can activate the transport of albumin (2, 6–8, 13, 19, 20, 23, 25, 28, 29). The signaling pathways mediating transcytosis are not well understood. The pathway involving the heterotrimeric G protein G_i and the activation of Src kinase signaling may activate albumin endocytosis and its transport from the luminal to the abluminal cell surface (12–14, 27). Interestingly, albumin transcytosis was augmented by antibody-induced cross-linking of gp60 (13, 27–29). These studies suggest that, under physiological conditions, albumin can bind to its cell surface receptors [the albumin-binding proteins (ABPs)] and initiate transcytosis.

However, important issues concerning the mode of albumin uptake and transport by a transcellular route remain unresolved. Fluid phase transport of albumin in vesicles or via the paracellular pathway would not be expected to be a saturable process, whereas transport of albumin, either bound to receptors or regulated by receptors, should be saturable. In addition, the high-affinity nature of ABPs such as gp60 (in the nanomolar range) (19, 25) relative to plasma and interstitial concentrations of albumin (<1 mM) implies that 1) albumin uptake and transcytosis should be constitutive processes and 2) bound albumin may not dissociate from its vesicular carriers upon exocytosis. These questions could not be previously addressed with any rigor, because determinations of affinity of albumin to the endothelial plasmalemma were hampered by high nonspecific binding (2, 6, 7, 19). This masked any saturable and specific albumin binding. In the present study, we determined the concentration dependency of albumin binding on the surface of confluent endothelial monolayers and transport of albumin. We specifically used cultured rat lung microvascular endothelial cells (RLMVEC), because they were shown by us to have low nonspecific cell surface adsorption of albumin. We show that 1) binding and uptake of albumin have saturable and fluid phase components, 2) cellular uptake of albumin is submaximal at physiological serum albumin concentration, implying that it can be further increased in response to appropriate signals, and 3) gp60, an ABP, can activate albumin transport.
METHODS

Endothelial Cell Cultures

RLMVEC (VEC Technologies, Rensselaer, NY) were cultured in high-glucose DMEM (GIBCO BRL, Grand Island, NY) supplemented with 5% fetal bovine serum (HyClone, Logan, UT) plus 50 U/ml penicillin and 50 μg/ml streptomycin, as described previously (4). The cultures were maintained in 5% CO₂-95% room air at 37°C.

Drugs and Reagents

All drugs and reagents were obtained from Sigma Chemical (St. Louis, MO) unless stated otherwise. Hank’s balanced salt solution (HBSS) containing NaHCO₃ (4.2 mM) and HEPES (10 mM) was adjusted to pH 7.4. Bovine serum albumin (fraction V, 99% pure, endotoxin free, cold alcohol precipitated) was freshly dissolved in HBSS in concentrations of 0.01–100 mg/ml. Methyl-β-cyclodextrin was dissolved in HBSS. Rabbit antibody against bovine endothelial gp60 (anti-gp60 antibody) was prepared and labeled with Cy3, as described elsewhere (25, 27). Control isotype-matched antibody was isolated from rabbit preimmune serum by precipitation (0.01 mg/ml). Rabbit antibody against bovine endothelial gp60. Controls were treated with the preimmune antibody (20 μg/ml) at 4°C for 30 min. Cells were rewarmed to 37°C to activate endocytosis (27). To deplete ABP gp60 (27), anti-gp60 antibody was preincubated with cells for 2 h at 37°C.

Albumin Iodination

Labeling of albumin with Na-125I (ICN Pharmaceuticals) was performed using chloramine T (3). The tracer albumin formed was purified to <0.4% free 125I using Sephadex G-25 columns (Sigma Chemical). Specific radioactivity was 0.3–1.0 μCi/μg albumin determined by 10% trichloroacetic acid precipitation.

Methyl-β-Cyclodextrin Treatment

Confluent RLMVEC monolayers were incubated with methyl-β-cyclodextrin (0.2 mM–10 mM) in HBSS for 15 min, washed twice with HBSS, and incubated with fresh medium containing tracer albumin for the desired periods.

Immunostaining

Cellular localization of albumin, gp60, and caveolin-1 in the plasma membrane and plasmalemmal vesicles exposed to albumin was determined by immunocytochemical labeling and laser scanning confocal microscopy (LSM 510, Zeiss). RLMVEC were serum deprived for 2 h, washed three times with HEPES-buffered HBSS or phenol red-free DMEM, and exposed to 0.1 mg/ml albumin in the presence of 50 μg/ml Alexa 488-conjugated albumin, 20 μg/ml cholora toxin subunit B-Alexa 594 conjugate, and cholora toxin subunit B-Alexa 594 conjugate were purchased from Molecular Probes (Eugene, OR).

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Cell Surface gp60 Cross-Linking

RLMVEC monolayers were washed twice with HEPES-buffered DMEM at 4°C and incubated for 30 min at 4°C with anti-gp60 antibody (20 μg/ml) and then with secondary antibody (goat anti-rabbit, 20 μg/ml) for 30 min to cross-link gp60. Controls were treated with the preimmune antibody (20 μg/ml) at 4°C for 30 min. Cells were rewarmed to 37°C to activate endocytosis (27). To deplete ABP gp60 (27), anti-gp60 antibody was preincubated with cells for 2 h at 37°C.

Binding of 125I-Albumin

Confluent cell cultures in six-well plates (1.0–1.2 × 10⁶ cells/35 mm) were washed twice in 10 mM HEPES-buffered DMEM, pH 7.4, and then serum deprived for 2 h by incubation in DMEM. Cells were precooled to 4°C for 30 min, and 125I-albumin in the presence or absence of unlabeled albumin in HBSS was added for 30 min at 4°C. Unbound ligand was removed by three washes with ice-cold HBSS, and cells were lysed with 1 ml of 50 mM Tris·HCl buffer, pH 7.4, containing 1% Triton X-100 and 0.5% SDS (13). Cell-associated 125I-albumin counts were measured in a gamma counter (Packard Instruments, Downers Grove, IL).

Uptake of 125I-Albumin

Serum-deprived confluent RLMVEC monolayers (see above) were pretreated with indicated reagents at 37°C for required periods before addition of 125I-albumin in the presence or absence of unlabeled albumin in HBSS. Uptake at 37°C was allowed to proceed for times indicated and then terminated by chilling on ice and washing three times with ice-cold HBSS. To remove cell surface-bound 125I-albumin, cells were washed three times with 1 ml of acid wash buffer (0.5 M NaCl and 0.2 M acetic acid, pH 2.5) (26). Cells were then lysed and counted as described above.

Transendothelial 125I-Albumin Transport

RLMVECs were grown on clear microporous polyester Transwell membranes (12 mm diameter, 1 cm² growth area, 0.4 μm pore size; Corning Costar, Cambridge, MA). The membrane inserts were filled with a total of 0.5 ml of incubate containing 125I-albumin in the presence or absence of unlabeled albumin. The lower well was filled with 1.5 ml of incubate of the same osmolarity as the inner well. Thus fluid levels and osmotic pressure in the “upper” and “lower” wells were equalized to minimize hydrostatic and osmotic effects. Labeled albumin and test agents were added to the upper well in equimolar solution, and transendothelial 125I-albumin permeability was measured at 37°C. Aliquots of 50 μl were sampled from the lower chamber every 10–15 min, and gamma radioactivity was measured. Albumin permeability was calculated for radiolabeled albumin flux across the cell monolayer as described elsewhere (5, 22). Trichloroacetic acid precipitation analysis and SDS-PAGE of the tracer albumin (27) in the abluminal chamber showed that the radioactivity measured on the abluminal side of the cell monolayer remained attached to albumin and that albumin was not hydrolyzed in the uptake or transport process.
layers grown on Transwell filter inserts received vehicle (without drug) or methyl-β-cyclodextrin (0.2 nM–10 mM) for 15 min in the upper chamber, and tracer albumin was added immediately thereafter. Methyl-β-cyclodextrin blocked transendothelial 125I-albumin permeability with an IC50 of ~1.0 μM (Fig. 2). The highest concentration of the inhibitor (10 mM) reduced permeability of the albumin tracer by ~80% (from 7.7 to 1.5 fmol·min⁻¹·10⁶ cells⁻¹), indicating the importance of the cellular pathway in albumin transport in RLMVEC.

Regulation of transendothelial albumin transport by gp60. Inasmuch as activation of gp60 can stimulate albumin endocytosis in endothelial cells via caveolae (13, 21, 27), we determined whether gp60 activation also increased the transport of tracer albumin. To prevent internalization of the antigen-antibody complex, gp60 on the apical cell surface was cross-linked, as described elsewhere (27), using an anti-gp60 antibody for 30 min in cold conditions (4°C). Cells were rapidly warmed to 37°C with a solution containing 125I-albumin (2.5 nM) plus unlabeled albumin (1.5 μM) to activate uptake of the tracer. Control cultures received isotype-matched antibody in lieu of anti-gp60 antibody. Cross-linking of gp60 significantly increased 125I-albumin permeability 2.3-fold above the control value of 38 ± 1.5 nL·min⁻¹·cm⁻². Excess unlabeled albumin (1 μM) reduced 125I-albumin permeability to 1.2 ± 0.6 nL·min⁻¹·cm⁻² (Fig. 3) in the cells subjected to gp60 cross-linking. To address the dependence of basal transport of tracer albumin on the cell surface gp60, RLMVEC monolayers were incubated with anti-gp60 antibody for 2 h at 37°C to deplete cell surface gp60 (9, 27). This procedure reduced basal transendothelial 125I-albumin permeability to 7.3 ± 0.6 nL·min⁻¹·cm⁻² (80% inhibition; Fig. 3).

Statistical Analysis

Data were analyzed by the nonlinear least-squares curve-fitting programs LIGAND (Elsevier Biosoft) and Microcal Origin (Microcal Software, Northampton, MA). Student’s t-test was used to compare results at significance level of P < 0.05. Multiple comparisons were made by ANOVA. All statistical tests were made using GraphPad Prism Instat software (San Diego, CA).

RESULTS

Albumin Transport in Endothelial Monolayers

Time course of transendothelial 125I-albumin transport. For studies of transendothelial 125I-albumin transport in the apical-to-basolateral direction, RLMVEC were grown to confluence on Transwell filter inserts (1-cm² surface area). Experiments began when the liquid in the upper well was replaced by a warm (37°C) solution containing 125I-albumin plus unlabeled albumin (1.5 μM or 0.1 mg/ml). During the 90-min period, aliquots of media (0.05 ml) in the lower well were sampled and analyzed for gamma radioactivity (see METHODS). Appearance of tracer albumin in the basal chamber vs. time described a straight line (Fig. 1), as predicted if the backflux of tracer (into upper chamber) was negligible. From the slope of the fitted line in Fig. 1, we calculated the 125I-albumin flux of 7.2 fmol·min⁻¹·10⁶ cells⁻¹. Excess unlabeled albumin (1.5 mM or 100 mg/ml) blocked 125I-albumin flux (Fig. 1), implying competition between labeled and unlabeled albumin at the apical endothelial plasmalemmal surface.

Cell pathway regulates albumin transport. The effect of methyl-β-cyclodextrin, the cholesterol-binding agent that disrupts caveolae (10), on transendothelial 125I-albumin permeability was assessed. RLMVEC mono-

Fig. 1. Time course of transendothelial 125I-albumin permeability. 125I-Albumin transport was determined in rat lung microvascular endothelial cells (RLMVEC) grown to confluence in Transwell inserts. Tracer albumin (68 nM 125I-albumin) was applied to the apical chamber together with 1.5 μM or 1.5 mM free albumin, which was also present in the basal chamber to equalize osmotic pressure, and samples from the basal chamber were taken every 10–15 min for 90 min (4 wells per incubation time). Specific transport of 125I-albumin (amount inhibited by 1.5 mM albumin) was >80% of total 125I-albumin clearance. 125I-Albumin transport was cumulative, increasing linearly for up to 90 min. Data are from 1 experiment, which is representative of 3 experiments performed.

Fig. 2. Inhibitory effect of methyl-β-cyclodextrin on flux of 125I-albumin. RLMVEC on Transwell inserts were preincubated for 15 min with 0.2 nM–10 mM methyl-β-cyclodextrin or vehicle and then with 125I-albumin in Hanks’ balanced salt solution (HBSS) containing 1.5 μM free albumin for 15 min. A dose-dependent inhibition of 125I-albumin flux from a control value of 7.7 ± 0.3 fmol·min⁻¹·10⁶ cells⁻¹ was observed. Values are means ± SE from 4 replicates for each point in 1 experiment, which is representative of 4 experiments performed.
Controls were treated with preimmune antibody (10 μg/ml) and incubated for 30 min with anti-gp60 antibody (10 μg/ml) and secondary antibody (goat anti-rabbit, 10 μg/ml) for 30 min to induce cross-linking. Controls were treated with preimmune antibody (10 μg/ml) and secondary antibody. Cells were rewarmed to 37°C to activate uptake of 125I-albumin and 1.5 μM unlabeled albumin. Cross-linking of gp60 increased 125I-albumin permeability 2.3-fold from a control value of 38 ± 15 nl·min⁻¹·cm⁻². To deplete cell surface gp60, RLMVEC monolayers were washed twice with HEPES-buffered DMEM and incubated with anti-gp60 antibody (10 μg/ml) for 2 h at 37°C. Depletion of gp60 reduced 125I-albumin transport by 85%. 125I-Albumin flux was also blocked by 95% when cells were coincubated with 1.5 mM unlabeled albumin (100 mg/ml). Values are means ± SE (n = 4). * P < 0.01 vs. control by ANOVA.

### Albumin Uptake in Endothelial Cell Monolayers

**Time course of tracer albumin uptake.** RLMVEC monolayers were incubated for various periods with 125I-albumin in the presence of 1.5 μM unlabeled albumin. 125I-albumin accumulation within the cells was determined by counting gamma radioactivity in cell lysates. Lysates were prepared from acid-washed cells to avoid contamination by tracer albumin associated with the cell exterior. The time course of cellular 125I-albumin uptake was biphasic (Fig. 4). Specific tracer uptake (125I-albumin uptake in the presence of 1.5 μM albumin minus that in 1.5 mM albumin) reached an initial peak (60 fmol/10⁶ cells) within 15 min, approached a minimum (50 fmol/10⁶ cells) at 30–45 min, and finally rose to a second peak (75 fmol/10⁶ cells) at 75 min. The initial slope of the uptake curve was used to estimate the rate of tracer albumin uptake, which was 6 fmol·min⁻¹·10⁶ cells⁻¹; this value was within the same range as 125I-albumin transendothelial transport of 7.2 fmol·min⁻¹·10⁶ cells⁻¹ (see above). Endothelial 125I-albumin uptake was markedly inhibited at each time point by excess (1.5 mM) unlabeled albumin (Fig. 4).

**Concentration dependence of albumin uptake.** Submaximal concentrations of unlabeled albumin produced biphasic inhibition of tracer albumin uptake (Fig. 5A; IC₅₀ = 1.15 μM). Scatchard analysis (Fig. 5B) used to parse the sigmoidal function into its two components showed a 0.87 μM high-affinity component, with maximal binding capacity (Bₘₐₓ) = 0.47 pmol/10⁶ cells, and a 93.3 μM low-affinity component, with Bₘₐₓ = 20.2 pmol/10⁶ cells. From these data, we determined the total albumin uptake (labeled + unlabeled forms) as a function of albumin concentration (Fig. 5C). The cellular uptake of albumin was dependent on albumin concentration; the steepest region of concentration dependency was between 10⁻⁵ and 10⁻³ M albumin. The smooth curve in Fig. 5C predicts an EC₅₀ of ~100 μM and saturation of transport at ~3 mM albumin.

**Confocal fluorescence imaging of albumin uptake.** We visualized the uptake of albumin in caveolae by colocalization of fluorescent forms of albumin, cholera toxin subunit B, and anti-gp60 antibody. Cholera toxin subunit B was used as a specific marker for caveolae, because it labels the caveolae-specific ganglioside GM1 (14); gp60 was previously shown to be associated with endothelial caveolae (13). After 30 min of incubation with these fluorescent probes at 37°C, cells were acid washed to remove residual cell surface probe, fixed, and stained with the nuclear marker DAPI (1 μg/ml) or anti-caveolin-1 antibody plus Alexa 350-labeled secondary antibody. High-resolution confocal images (<1.0 μm optical thickness) in Fig. 6A show internalized Alexa 488-albumin and Cy3-gp60 antibody, together with caveolin-1 immunostaining near the apical cell surface. Coincident staining of gp60 and albumin and colocalization of all three probes were apparent in the overlay. In other experiments, fluorescent albumin was coincubated with fluorescent cholera toxin subunit B. Figure 6B shows the confocal images of internalized Alexa 594-cholera toxin subunit B and Alexa 488-albumin, together with DAPI. The fluorescence of albumin and cholera toxin was coincident in the merged image, indicating albumin’s association with caveolae.

To address the effects of methyl-β-cyclodextrin on albumin uptake and caveolin-1 distribution, endothelial cells were pretreated with 10 mM methyl-β-cyclodextrin and incubated with HBSS containing Alexa...
Analysis of combined tracer 125I-albumin plus unlabeled albumin binding capacity. Data are representative of 4 separate experiments. Increasing concentrations of unlabeled albumin (10^{-3} M) were added to RLMVEC monolayers followed by 34 nM 125I-albumin, and monolayers were incubated at 37°C for 1 h. A: uptake of 125I-albumin was inhibited in the presence of increasing concentrations of free albumin. IC50 was calculated to be 1.15 μM; near-maximal displacement was achieved with 1 μM albumin. B: Scatchard analysis of 125I-albumin displacement was best fit by a 2-site model, represented by $K_D$1 and $B_{\text{max}1}$ (site 1) and $K_D$2 and $B_{\text{max}2}$ (site 2), where $K_D$ is affinity constant and $B_{\text{max}}$ is maximal binding capacity. C: total albumin uptake calculated from Scatchard analysis of combined tracer 125I-albumin plus unlabeled albumin uptake shows saturation of albumin uptake with ~3 mM added albumin. Data are representative of 4 separate experiments.

**Table 1**

<table>
<thead>
<tr>
<th>Site</th>
<th>Affinity Constant</th>
<th>Maximal Binding</th>
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<tbody>
<tr>
<td>1</td>
<td>13.5 nM</td>
<td>24.2 fmol/10^6 cells</td>
</tr>
<tr>
<td>2</td>
<td>1.6 μM</td>
<td>2.4 pmol/10^6 cells</td>
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**DISCUSSION**

RLMVEC monolayers exhibited a high degree of specific 125I-albumin binding, because >80% of total binding was inhibited by excess unlabeled albumin. Although a saturable low-affinity binding site for albumin has been described in bovine pulmonary artery endothelial cells (23) and rat fat tissue microvessel endothelial cells (20), the finding of a second site of much higher affinity is a novel observation. Competition studies of 125I-albumin binding under cold conditions (4°C) to prevent tracer internalization showed that albumin bound to the endothelial cell surface with affinity constants of 13.5 nM ($K_D$1) and 1.6 μM ($K_D$2). On the basis of the corresponding $B_{\text{max}}$ values, the high-affinity site represented ~1% of the specific albumin binding and averaged 5 × 10^3 sites/cell. Schnitzer and Oh (21) and Tiruppathi et al. (25) demonstrated that an anti-gp60 antibody displaced the specific binding of 125I-albumin from rat and bovine pulmonary microvascular endothelial cells, consistent with our
Fig. 6. Colocalization of albumin with gp60, cholera toxin subunit B, and caveolin-1. A: high-magnification confocal image (2-μm optical thickness) of RLMVEC shows Alexa 488-albumin uptake (green, bottom left), Cy3-gp60 antibody uptake (red, top left), and caveolin-1 immunostaining (blue, top right) near the apical surface. Overlay of gp60, albumin, and caveolin-1 images (bottom right) shows coincident staining of gp60 and albumin as yellow and as white where gp60 and albumin are closely associated with caveolin-1 (red, green, and blue). B: confocal image shows confluent RLMVEC monolayer staining with Alexa 594-cholera toxin subunit B (red, top left), Alexa 488-albumin (green, bottom left), and DAPI for nuclear localization (blue, top right). Note colocalized fluorescence of albumin and cholera toxin in merged image (yellow; bottom right), suggesting albumin uptake by caveolae. Images are representative of 4 experiments.
observations. Thus the specific binding sites for albumin may include gp60.

Because the measured albumin uptake only represents that albumin tracer internalized by the cells (which thus was present in the lysate from acid-washed cells), it is likely that uptake data report the endocytosis of the tracer. At $10^{-6}$–$10^{-3}$ M unlabeled albumin, we observed progressive inhibition of tracer albumin uptake (Fig. 5A). From Scatchard analysis (Fig. 5B), we determined the apparent albumin binding constants of the transporter to be 0.87 $M^{-1}$ (low affinity) and 93 $M^{-1}$ (ultra-low affinity). The low-affinity uptake value is similar to other reported low-affinity binding values (20, 23). The low-affinity albumin binding may represent the tracer albumin, which was initially bound to a saturable site at the cell surface such as gp60 and was subsequently internalized by endocytosis. By contrast, the ultra-low-affinity component may represent tracer albumin sequestered within a restricted membrane compartment (with limited access to the extracellular fluid). Endothelial caveolae, which communicate with the extracellular fluid via narrow (20- to 30-nm) necks (17), could qualify as such a compartment. Our evidence for this is as follows: 1) at 4°C when internalization by endocytosis is completely inhibited, the ultra-low-affinity binding component was not detected (Fig. 8); 2) at 37°C, methyl-$\beta$-cyclodextrin, which was shown to eliminate caveolae (Fig. 7), markedly inhibited all components of $^{125}$I-albumin uptake (Fig. 2); and 3) Alexa 488-albumin entered the caveolar compartment, because the fluorophore was shown to colocalize in endocytic vesicles with Cy3-labeled anti-gp60 antibody (Fig. 6A) and Alexa 594-cholera toxin subunit B (Fig. 6B), the specific caveolae marker (15).

We used methyl-$\beta$-cyclodextrin as a reagent to address the relationship between albumin uptake and transport. Methyl-$\beta$-cyclodextrin abolished albumin uptake and transport (Figs. 2 and 7). The albumin transport protocol monitored the transfer of albumin tracer through the cell monolayer and into the abluminal chamber. In this assay, the backflux of tracer albumin from the abluminal compartment was minimal because of dilution of the volume in the abluminal well, thus allowing a measure of unidirectional albumin flux. Moreover, we imposed no transendothelial hydrostatic or oncotic pressure gradient, thus rendering negligible the convective movement of tracer. We infer from the methyl-$\beta$-cyclodextrin sensitivity of transendothelial albumin transport that the diffusional paracellular flux of tracer albumin in RLMVEC was minimal (Fig. 2).

The earliest step in the uptake and transport of albumin through endothelial cells is the “docking” of albumin on the caveolae-associated ABPs (2, 6, 7, 13, 19–21, 25, 28, 29). Inasmuch as the transendothelial flux of tracer albumin was abolished by excess unlabeled albumin (1.5 mM), the albumin internalized by endothelial cells and transported through the monolayer is displaceable (Fig. 1). In the presence of a much
lower concentration of unlabeled albumin (1.5 μM), there is a linear relationship between albumin transport and time (Fig. 1). From the slope of this relationship, we calculated the rate of tracer albumin transport to be 7.2 fmol albumin·min⁻¹·10⁶ endothelial cells⁻¹. Importantly, we observed that the tracer albumin uptake rate of 6 fmol·min⁻¹·10⁶ cells⁻¹ (calculated from the initial slope of the uptake curve in Fig. 4) was comparable to the transport rate.

Analysis of the time course of tracer albumin uptake showed peaks at 15 and 75 min (Fig. 4). A possible interpretation of this complex uptake process is that this represents the reuptake of albumin tracer that has been exocytosed. Recycling of the tracer involving uptake, extrusion, and reuptake could account for the dual uptake peaks.

Anti-gp60 antibody exposure of cells used to deplete the cell surface gp60 (27) reduced albumin uptake and transport in the endothelial barrier at 37°C by ~85% (Fig. 3). This finding is consistent with a role of cell surface gp60 in mediating albumin transport via transcytosis. By contrast, gp60 activation induced by a much briefer exposure to the anti-gp60 antibody plus a secondary antibody (to cross-link gp60 (27)) markedly increased the transport of ¹²⁵I-albumin. This method of cross-linking is analogous to that induced in activating growth factor receptors, which dimerize, or glycosylphosphatidylinositol-anchored proteins, which cluster in rafts or caveolae, on exposure to specific antibodies (1). Because cross-linking by the anti-gp60 antibody increased albumin uptake and transport at 37°C but blocked albumin binding at 4°C, it is an activating and blocking antibody, depending on the temperature.

The data show that the antibody can compete with albumin for binding to gp60 at 37°C as well as 4°C. However, at 37°C, ~98% of the internalized albumin is in the fluid phase compartment of plasmalemma vesicles, rather than “receptor bound,” because it required significantly more unlabeled albumin to antagonize competitively ¹²⁵I-albumin uptake (Fig. 5A) than ¹²⁵I-albumin binding (Fig. 8A). The data in Fig. 5B show that the ultra-low-affinity uptake of albumin has a much greater Bmax (20.2 pmol/million cells) than the low-affinity component (0.47 pmol/10⁶ cells), suggesting that fluid-phase endocytosis represents ~98% of the overall uptake of albumin in RLMVEC.

![Diagram](image)

**Fig. 9. Model of gp60-mediated albumin transport based on the presented data.** Under basal, albumin-free conditions, gp60 exists as a high-affinity (Kₐ = 10 nM) cell surface albumin-binding protein or receptor; on activation by albumin, gp60 clusters, resulting in a 100-fold decrease in albumin affinity (K_D2 = 1 μM; i). Phosphorylation stimulated by gp60 activation may result in caveolin-1 oligomerization (17, 13, 27) and fission of caveoleae from the membrane; fluid-phase component of vesicular albumin has a 100-fold lower affinity (K_D3 = 100 μM) than gp60-bound albumin (ii). Release of caveoleae from the plasma membrane traps fluid-phase albumin and other plasma constituents within the vesicle; fluid phase accounts for 98% of the albumin internalized (iii).
increased albumin transport induced by gp60 cross-linking appears to be primarily the result of increased fluid-phase albumin transport subsequent to activation of endocytosis.

The model (Fig. 9) predicts that albumin transport can increase on the basis of activation of gp60 and the internalization of caveolae carrying albumin in the fluid phase. As shown in the model, albumin binding to the high-affinity cell surface gp60 induces clustering of this “receptor” and its association with caveolin-1. Clustered gp60 has a reduced affinity for albumin (in the 1 μM range) that may represent the physiological state of gp60, because it is constantly exposed to saturating concentrations of albumin. Clustering of gp60 may, through activation of specific signaling pathways dependent on Gαi and Src (12, 13, 27), induce the release of caveolae from the luminal plasma membrane. Inasmuch as caveolae bud from the membrane in this model, fluid-phase albumin is trapped within these released vesicles. According to our measurements, albumin uptake by this ultra-low-affinity (in the range 100 μM), high-capacity system represents 98% of total internalized albumin.

What is the previous evidence in favor of transcytosis of albumin in the endothelial cell monolayer? In a series of studies, gp60 activation was shown to stimulate apical-to-basolateral migration of vesicles as monitored using interfacial fluorescent probes RH414 and FM1–43 (12, 13). Activation of gp60 also increased the transport of albumin and horseradish peroxidase without altering the hydraulic conductivity or decreasing transendothelial electrical resistance in bovine endothelial cells (13, 27). These findings suggest transport of albumin via nonhydraulic or transcellular pathways utilizing vesicle carriers. Studies also showed (28), using distinct interfacial and paracellular probes, that gp60 activation selectively increased the flux of the interfacial probes, suggesting activation of plasmalemmal vesicle trafficking.

In summary, our data support the hypothesis that J) transendothelial albumin transport in the RLMVEC used in the present study is dominated by the transcytosis pathway, 2) this mode of transport is saturable, 3) binding of albumin to a limited number of high-affinity sites on the endothelial cell may activate albumin transport by the release of caveolae from the membrane, and 4) the bulk of the albumin within the transport vesicles is in the fluid phase. Inasmuch as the specific binding sites for albumin on the endothelial cell surface are high affinity relative to the physiological concentrations of serum and interstitial albumin, this implies that albumin transcytosis should be continuously active. This is evident for the numerous vesicles seen in resting endothelial cells in situ that comprise ~20% of the cell volume (17). However, as our data demonstrate, albumin uptake is not saturated at physiological concentrations of albumin. Rather, the uptake is maximal in the presence of ~3 mM albumin, which is five times greater than plasma albumin concentration, suggesting the potential for increased albumin transport via transcytosis in response to activation of specific signaling pathways.

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