Gp60 Activation Mediates Albumin Transcytosis in Endothelial Cells by Tyrosine Kinase-dependent Pathway*

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We investigated the function of gp60, an endothelial cell membrane 60-kDa albumin-binding protein localized in caveolae, and the mechanism of its activation in regulating endothelial permeability of albumin. Gp60 organization on the bovine pulmonary microvessel endothelial cell (BPMVEC) surface was punctate as shown by immunofluorescence using an anti-gp60 antibody (Ab) conjugated with bifunctional, N-hydroxysuccinimidyl fluorophore (Cy3). Addition of a secondary Ab to anti-gp60 Ab-treated BPMVEC induced cross-linking of gp60 as evident by increased size of fluorescent particles and cell surface gp60 clustering. Gp60 cross-linking also produced 2–3-fold increases in the endothelial cell uptake and the luminal to abluminal permeability of 125I-albumin as well as the fluid-phase tracer, horseradish peroxidase. The increased transendothelial permeability of macromolecules was the result of transcytosis as it was not associated with an increase in the paracellular pathway. Incubation of anti-gp60 Ab with BPMVEC at 37 °C caused internalization of gp60, and thereby reduced the uptake of the macromolecules. Activation of gp60 by either albumin (the gp60 ligand) or gp60 cross-linking induced the phosphorylation of both gp60 and caveolin-1 (the major structural caveolar protein) on tyrosine residues. Gp60 activation also phosphorylated the Src family tyrosine kinases pp60<sup>Src</sup> and Fyn. The activated pp60<sup>Src</sup> and Fyn co-immunoprecipitated with caveolin-1 in BPMVEC membrane. Protein tyrosine kinase (PTK) inhibitors, herbimycin A and genistein, prevented gp60-activated macromolecule uptake and transcytosis in a concentration-dependent manner, indicating the functional significance of the PTK pathway in activating albumin transcytosis. These findings indicate that activation of gp60 stimulates the Src PTK signaling pathway, and thus regulates the transcytosis of albumin across the endothelial cell monolayer.

Serum albumin maintains the transendothelial oncotic pressure gradient (1–4) and regulates the transport of fatty acids, steroids, thyroxine, and amino acids because of albumin’s unique binding properties (1–4). Albumin, through its interaction with matrix components of the endothelial cell membrane, also serves a critical function in maintaining the characteristic permselective endothelial cell monolayer barrier (5). Morphological studies using gold-conjugated albumin tracer have shown that albumin can bind to the endothelial cell surface resulting in the localization of the albumin tracer in plasmalemmal vesicles followed within 30–60 min by deposition of the tracer in the abluminal space (6–9).

Albumin-binding proteins (ABPs) identified on the endothelial cell surface have been suggested to regulate the transcytosis of albumin (6–9). Ghinea et al. (6) observed in ligand blotting and photochemical cross-linking studies that albumin could bind specifically to 18- and 31-kDa polypeptides in fat tissue and lung capillary endothelial cells. Conformationally modified albums (i.e. albumin-Au, formaldehyde-, or maleic anhydride-treated albumin) were shown to bind preferentially with a 1000-fold greater affinity to these proteins compared with monomeric native albumin (10, 11). Studies using lectins also identified a 60-kDa (gp60) ABP on the endothelial cell membrane which binds specifically to native albumin (12). The lectins Limax flavus agglutinin and Ricinus communis agglutinin inhibited albumin binding to rat fat tissue microvessel endothelial cells by preventing the binding of albumin to gp60 (12). We showed that R. communis agglutinin precipitated gp60 from endothelial cell membranes and inhibited transendothelial albumin transport by ~50% (13), suggesting an important function of gp60 in regulating albumin permeability.

Antibodies (Abs) raised against purified gp60 (14) prevented albumin binding to the endothelial cell surface at 4 °C. The membrane-bound gp60 has several unique characteristics, among them its difference from the endothelial cell-secreted ABP, secreted protein, acidic and rich in cysteine (14). Since the function of gp60 in regulating vascular endothelial permeability is poorly understood, in the present study, we have determined the function of gp60 in mediating the uptake of albumin and its transport via a transcellular mechanism. We have also studied the role of the protein tyrosine kinase pathway in signaling the gp60-activated albumin transport across the endothelial cell monolayer.

EXPERIMENTAL PROCEDURES

Endothelial Cell Culture—Bovine pulmonary microvessel endothelial cells (BPMVEC) were isolated and cultured as described (15). Endothelial cells were grown in Dulbecco’s modified essential medium supplemented with 10% fetal bovine serum. Confluent endothelial monolayers were incubated in serum-free medium for 15 h to deplete the albumin. Cells passed between 19 and 22 times were used in the experiments described below.

Cell Surface Gp60 Immunofluorescence—Polyclonal antiserum against gp60 was prepared and purified as described (14). Anti-gp60 Ab was labeled with Cy3 bifunctional reactive dye (Amersham Life Science, Inc., Pittsburgh, PA) according to the manufacturer’s instructions.

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BPMVEC cultured on glass coverslips were incubated with serum-free medium overnight at 37 °C, and then incubated with 5% horse serum in phosphate-buffered saline for 30 min at 4 °C, and washed 3 × with Hank’s balanced salt solution at 22 °C. The cells were incubated with anti-gp60 Ab-Cy3 (50 μg/ml) for different time intervals, and washed 3 times with Hank’s balanced salt solution at 22 °C. The coverslips were fixed with mounting fluid and viewed on a Zeiss IM-35 fluorescence microscope ×40 objective. Micrographs were taken on T-max 400 film (Eastman Kodak) using identical exposure times.

RESULTS

Cell Surface Localization of Gp60—The anti-gp60 Ab-Cy3 binding patterns on BPMVEC revealed a punctate cell surface organization in the absence of nuclear labeling (Fig. 1). The particle size was variable and the brightness increased with the time of incubation with the fluorophore (Fig. 1, A-C). Staining was competitively inhibited by unlabeled anti-gp60 Ab (data not shown). Maximum brightness was observed within 30 min of incubation, and thereafter, the fluorescence intensities remained unchanged. Addition of a secondary Ab (goat anti-rabbit IgG) to induce gp60 cross-linking significantly increased the size of fluorescent particles compared with controls (Fig. 1D).

Endothelial Cell Monolayer Electrical Impedance—The method of determining endothelial monolayer impedance has been previously described by us for assessment of the paracellular transport pathway (21). We used this measurement to determine whether the increase in 125I-albumin and HRP transport was the result of endothelial cell retraction, and opening of intercellular gaps. BPMVEC were grown to confluence on a 4.9 × 10^{-4} cm^{-2} gold electrode. The small electrode and the larger counter electrode were connected to a phase-sensitive lock-in amplifier (21). An constant current of 1 μA was applied by a 1 V, 4000 Hz AC signal connected serially to 1 MΩ resistor between the small electrode and the larger counter electrode. The voltage between small electrode and larger counter electrode was continuously monitored by lock-in amplifier, stored, and processed on a computer. The data are presented as change in the resistive portion of the impedance normalized to its value at time 0 as described (21).

Immunoblotting of BPMVEC with Anti-gp60 Ab-Cy3 and Anti-Fyn Ab—BPMVEC grown to confluence on 6.5-mm diameter filters were incubated with lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 0.2% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM Na3VO4, 1 mM sodium fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml aprotinin, and 44 μg/ml phenylmethylsulfonyl fluoride) at 4 °C for 30 min. The lysates were separated on SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with blocking buffer (5% dry milk in 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl) for 2 h at 22 °C. The membrane was washed and incubated with either anti-p60c-Src monoclonal Ab (from Oncogene Research Products, Cambridge, MA) or anti-Fyn monoclonal Ab (from Transduction Laboratories, Lexington, KY) in blocking buffer overnight at 4 °C. The membranes were washed and the reactive proteins were identified after incubating with 125I-goat anti-mouse IgG. The protein bands were identified using known molecular weight markers.

Phosphorylation of p60c-Src and Fyn—BPMVEC grown in 60-mm culture dishes were washed and incubated with serum-free medium for 15 h. Then the cells were washed and incubated with DMEM containing 10% fetal bovine serum and 1 μCi/ml [3H]Frophosphate (1 μCi/ml) for 4 h. After this incubation period, the cells were stained with either albumin or anti-gp60 Ab. The cells were washed and lysed with 1 ml of lysis buffer for 4 min at 30 °C. The cell lysate was collected and centrifuged at 13,000 × g for 10 min and the clear supernatant was incubated with either anti-p60c-Src Ab or anti-Fyn Ab overnight at 4 °C. Then the samples were incubated with protein G-Sepharose beads for 1 h to separate precipitated proteins. The beads were washed 4 times with wash buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% Triton X-100, 1 mM Na3VO4, and 1 mM sodium fluoride). The washed beads containing the precipitated proteins were separated on SDS-PAGE and autoradiographed.

Phosphorylation of Caveolin-1 and Gp60—The experimental protocol used was similar to the described above except that the cell lysate was immunoprecipitated using anti-caveolin Ab (from Transduction Laboratories, Lexington, KY) or anti-gp60 Ab.

Tyrosine Phosphorylation of Gp60—BPMVEC grown to confluence in 60-mm dishes were washed and incubated with serum-free medium for 15 h and then treated with test components. The cells were washed, lysed with lysis buffer, and the lysate was incubated with anti-gp60 Ab at 4 °C. After this incubation, the immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the beads were washed 4 times with wash buffer. The precipitated proteins were separated on SDS-PAGE, transferred to nitrocellulose membrane, and blocked with 2.5% BSA in TBS (20 mM Tris, 150 mM NaCl, pH 7.5) containing 1 mM Na3VO4 and 1 mM sodium fluoride for 60 min at 22 °C. The membrane was washed twice with 0.05% Tween 20 containing TBS. The membrane was then incubated with anti-phosphoarginine monoclonal Ab (from ICN Biochemicals, Costa Mesa, CA) diluted in blocking buffer for 12 h at 22 °C. The membrane was washed and incubated with 125I-goat anti-mouse IgG for 60 min at 22 °C. The protein bands were visualized by autoradiogram. In some experiments, anti-phosphoarginine Ab-blotted membrane was stripped at 50 °C for 30 min with 70 mM Tris-HCl, pH 6.8, containing 2% SDS and 2 mM β-mercaptoethanol, washed, and then used for probing with another Ab.

RESULTS
observed at 30 min (Fig. 2). Incubation of the monolayers for 60 min decreased the uptake of BSA. These results cannot be attributed to 125I-BSA degradation following its internalization in endothelial cells since analysis by SDS-PAGE of BSA recovered from the incubation medium and cell lysate indicated that BSA remained intact in the medium and cell lysate (data not shown).

We studied whether other macromolecules in the fluid phase could also be internalized by gp60 cross-linking. The uptake of HRP using the medium containing 1 mg/ml unlabeled BSA and 0.4 mg/ml HRP was studied as described above for 125I-BSA. Gp60 cross-linking increased the uptake of HRP by 2–3-fold compared with control cells (Fig. 3). HRP uptake was 5–6-fold greater in the presence of BSA than its absence, suggesting that albumin binding to gp60 facilitated the fluid phase HRP uptake (data not shown).

**Association of Gp60 Internalization with Loss of Albumin Uptake**—We preincubated BPMVEC monolayers with anti-gp60 Ab for 30 min at 37 °C and washed 3 times with pH 3.0 buffer at 4 °C to remove the cell surface-bound Ab. The monolayers were treated with anti-gp60 Ab and secondary Ab for 30 min at 4 °C, and used for 125I-BSA uptake as described above. The 125I-BSA uptake did not significantly increase in these monolayers (Fig. 4A).

Endothelial cell monolayers were incubated with anti-gp60 Ab at 37 °C for different time intervals, placed on ice, washed with ice-cold acid buffer, pH 3.0, to remove surface-bound Abs, and incubated with anti-gp60 Ab-Cy3 for 30 min at 22 °C. The fluorescent particle number on the cell surface was reduced in cells treated with anti-gp60 Ab at 37 °C for 30–60 min (Fig. 4B), a finding consistent with internalization of the cell surface gp60 and the reduction in 125I-BSA uptake (Fig. 4A).

**Endothelial Cell Surface Gp60 Cross-linking Activates Transcytosis of Macromolecules**—BPMVEC were cultured on micro-

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**Fig. 1. Immunofluorescence localization of gp60 on the endothelial cell surface.** BPMVEC cultured on glass coverslips were incubated with anti-gp60 Ab-Cy3 for 5 min (A), 15 min (B), and 30 min at 22 °C. In D, the cells were first treated with anti-gp60 Ab-Cy3 for 30 min and then incubated with secondary Ab (goat anti-rabbit IgG) for 20 min. The cells were washed, fixed with mounting a fluid, and observed using × 40 objective. Details are described under “Experimental Procedures.” The results are representative of five experiments.

**Fig. 2. Effects of cross-linking gp60 on 125I-albumin uptake in BPMVEC.** The cells were incubated with either anti-gp60 Ab or control Ab (100 μg/ml) for 30 min at 4 °C, washed, and incubated with goat anti-rabbit IgG for 20 min at 4 °C. Endothelial cell monolayers were washed twice at 4 °C and used for tracer uptake at 37 °C for different time intervals. The uptake medium (1 ml) contained 1 mg of BSA and 0.5 μCi of 125I-BSA. After incubation, the cells were washed 3 times with pH 2.5 buffer at 4 °C to remove surface-bound BSA. Details are described under “Experimental Procedures.” The results are shown mean ± S.E. for three separate experiments made in triplicate assays. * indicates the difference from control (p < 0.001). ●, control; ■, control Ab; ■, anti-gp60 Ab.

**Fig. 3. Effects of cross-linking gp60 on HRP uptake in endothelial cells.** BPMVEC monolayers were washed and incubated with anti-gp60 Ab and secondary Ab as described in the legend to Fig. 2. The uptake medium contained BSA (1 mg/ml) and HRP (0.4 mg/ml). Details are described under “Experimental Procedures.” The results are shown as mean ± S.E. for three experiments carried out in triplicate. *, indicate the difference from control (p < 0.001).
porous filters consisting of luminal and abluminal chambers as described under “Experimental Procedures” to measure the permeabilities of $^{125}$I-BSA and HRP. Endothelial monolayers were treated with anti-gp60 Ab at 22 °C, and permeability was measured at 37 °C. Gp60 cross-linking, increased the transendothelial $^{125}$I-BSA and HRP clearance rates by 2-fold in BPMVEC monolayers over control monolayers (Fig. 5, A and B).

We also studied whether gp60-induced increases in the paracellular pathway could account for the increase in endothelial permeability of macromolecules (21). BPMVEC cultured on gold electrodes were treated either with control Ab or anti-gp60 Ab, and then with the secondary Ab. Gp60 cross-linking did not...
orthophosphate, and BSA was added to the cells for dif-
ficulties. The anti-gp60 Ab or control Ab treated endothelial cells were again treated with secondary Ab (position of II arrow). The curves represent the reductive portion of the endothelial monolayer impedance normalized to its value at time 0. Results are representative of four separate experiments.

Gp60 Activation induces Src Phosphorylation—Because activation of Src kinases may represent an early signal induced by gp60, we studied the effects of gp60 activation on the activity of pp60\textsuperscript{Src} and Fyn. Immunoblot analysis of BPMVEC lysate using monoclonal Abs to pp60\textsuperscript{Src} and Fyn indicated that pp60\textsuperscript{Src} and Fyn were expressed in BPMVEC (Fig. 7A). We studied whether gp60 activation could mediate the phosphorylation of these tyrosine kinases in endothelial cells. BPMVEC monolayers were metabolically labeled with \textsuperscript{32}Porthophosphate (see “Experimental Procedures”) and stimulated with varying concentrations of BSA for 10 min at 37 °C. The cells were washed, lysed, and immunoprecipitated with either anti-pp60\textsuperscript{Src} Ab or anti-Fyn Ab after stimulation. The immunoprecipitated proteins were separated on SDS-PAGE and autoradiographed. Results indicated that BSA induced the phosphorylation of both pp60\textsuperscript{Src} and Fyn (Fig. 7B and 7C). Moreover, anti-pp60\textsuperscript{Src} and Fyn Abs co-immunoprecipitated two other major phosphorylated proteins (21–23 and 36 kDa) in the BSA-stimulated cells. We identified the 21–23 kDa protein by immunoblot as caveolin-1, a known substrate for Src family kinases (22, 23). The 36-kDa protein may be the low density lipoprotein receptor (CD 36) known to be associated with Src kinases (23, 24). The phosphorylation pp60\textsuperscript{Src} and Fyn induced by gp60 cross-linking in BPMVEC was similar to that observed with BSA stimulation (Fig. 7D).

Gp60 Activation Phosphorylates Gp60 and Caveolin-1 in Endothelial Cells—To assess the relationship between PTK signal-
ing and gp60 activation, we determined whether gp60 and caveolin-1 were phosphorylated following gp60 activation. BPMVEC monolayers were metabolically labeled with \textsuperscript{32}Porthophosphate, and BSA was added to the cells for different time intervals, washed, lysed, and immunoprecipitated with either anti-gp60 Ab or anti-caveolin-1 Ab. BSA (the gp60 ligand) induced the time-dependent phosphorylation of both gp60 and caveolin-1 (Fig. 8, A and B). We incubated the BPMVEC with serum-free medium and treated with BSA for 10 min, and the cells were washed, lysed, immunoprecipitated with anti-gp60 Ab, and blotted with anti-phosphotyrosine monoclonal Ab (see “Experimental Procedures”). Results indicated that BSA induced tyrosine phosphorylation of gp60 in a concentration-dependent manner (Fig. 8C). The anti-phosphotyrosine Ab-blotted membrane was stripped and blotted with anti-gp60 Ab to determine whether the anti-gp60 Ab immunoprecipitated equal amounts of gp60 from the cell lysate. These results indicated that gp60 precipitated by the Ab was similar in each case (Fig. 8D).

PTK Inhibitors Prevent Gp60-activated Macromolecule Uptake in Endothelial Cells—Because Src kinases were activated by gp60, we studied the effects of tyrosine kinase inhibitors, herbimycin A and genistein, on 


duced by gp60 cross-linking and in control cells (Fig. 9, A and B). Both herbimycin A and genistein also produced a similar inhibitory effect on transendothelial flux of albumin in gp60-activated endothelial cells (data not shown). In contrast, calphostin C (protein kinase C inhibitor (25)) did not alter gp60-activated macromolecule uptake in BPMVEC (Fig. 9B, inset).

DISCUSSION

Several ABPs have been identified on the endothelial cell membrane; however, their function in regulating the transen-
dothelial permeability of albumin remains unclear (6–14). The 60-kDa ABP (gp60) has been suggested to a critical ABP mediating the transport of albumin from the luminal to abluminal surface of the endothelial cell barrier (12–14). We have developed a method for purifying gp60 from endothelial cell membranes and have shown that Abs raised against bovine gp60 inhibits the specific binding of albumin to BPMVEC at 4 °C (14). We employed the strategy of cross-linking the endothelial cell surface gp60 using the anti-bovine gp60 Ab to study the function of gp60 activation in regulating both the uptake and transendothelial permeability of albumin.

Immunofluorescence imaging of gp60 on the BPMVEC surface (using the anti-gp60 Ab conjugated to the fluorophore Cy3) revealed a punctate gp60 distribution on the cell membrane (Fig. 1). Addition of a secondary Ab (goat anti-rabbit IgG) produced discrete cell membrane patches characteristic of clustering of cell surface receptors (26–28). Binding of hormones (e.g. platelet-derived growth factor) to their receptors induces a similar receptor clustering effect (29, 30). Clustering is typically followed by internalization of the ligand-receptor complex in endocytic vesicles where the complex is directed to lysosomal pathways for degradation (29, 30). Clustering of receptors induced by cross-linking also activates intracellular signaling events (30–33); for example, cross-linking of CD18 integrins on neutrophil plasma membrane with primary and secondary Abs caused generalized neutrophil activation similar to that observed with the binding of CD18 to its ligand, ICAM-1 (i.e. increase in free intracellular Ca\textsuperscript{2+}, exocytosis of azurophilic granules, up-regulation of CD18, shedding of i-selectin, and actin polymerization).

Because cross-linking induced endothelial cell surface gp60 clustering, we studied the possibility that gp60 activation by this mechanism would promote the uptake and transport of albumin across the endothelial cell monolayer. We used cultured BPMVEC monolayers because we have shown that these cells have an abundant number of vesicles whose pattern and distribution resemble in situ characteristics of endothelial cells.
lining pulmonary microvessels (19). We observed 2–3-fold increases in the uptake and transport of 125I-labeled albumin in BPMVEC following gp60 cross-linking (Figs. 2 and 5). Analysis of BSA recovered from the incubation medium and cell lysate by SDS-PAGE indicated that BSA remained intact in the medium and cell lysate, suggesting that albumin was transported into non-lysosomal transcytotic pathways where it avoided degradation.

The increase in transendothelial albumin permeability activated by gp60 cannot be ascribed to increase in transendothelial albumin flux occurring through the paracellular pathway because gp60 cross-linking increased the uptake of macromolecules without changing the transendothelial electrical impedance (Fig. 6). Therefore, the paracellular pathway did not increase as is the case with permeability-increasing mediators such as thrombin and histamine (21, 34). The present results are consistent with the hypothesis that albumin binding to the endothelial cell surface gp60 triggers a receptor-mediated mechanism of albumin transcytosis.

membrane was blotted with either anti-pp60c-Src monoclonal Ab or anti-Fyn monoclonal Ab. The reactive protein bands were identified after incubating with 125I-goat anti-mouse IgG. Both Abs reacted with the estimated 59–60-kDa protein. In B and C, BPMVEC were incubated with DMEM containing 180 μM inorganic phosphate and 100 μCi/ml [32P]orthophosphate for 4 h at 37 °C. The cells were then incubated with 1.0, 5.0, and 10.0 mg/ml BSA respectively. In C, BPMVEC monolayers were incubated with varying concentrations of BSA for 10 min and then the monolayers were washed and lysed. The lysates were immunoprecipitated with anti-gp60 Ab and the proteins were resolved on SDS-PAGE and transferred to nitrocellulose membrane. The membrane was immunoblotted with anti-phosphotyrosine monoclonal Ab. Lane 1, control; lanes 2–4, incubated with 0.1, 1.0, and 5.0 mg/ml BSA, respectively. In D, the same anti-phosphotyrosine blot in C was stripped and blotted with anti-gp60 Ab to indicate equal amounts of gp60 protein present. Other details were described under “Experimental Procedures.” The results are representative of three experiments.

FIG. 7. Gp60 activation induces phosphorylation of pp60c-Src and Fyn in BPMVEC. In A, BPMVEC grown in 60-mm tissue culture dishes were washed and lysed with lysis buffer (see details under “Experimental Procedures”). Endothelial cell lysate proteins were separated on SDS-PAGE and transferred to nitrocellulose membrane. The

FIG. 8. Binding of albumin to gp60 induces phosphorylation of gp60 and caveolin-1 in BPMVEC. BPMVEC monolayers were incubated with 100 μCi/ml [32P]orthophosphate for 4 h and then 5 mg/ml BSA was added and incubated for different time intervals at 37 °C. The cells were washed, lysed, and immunoprecipitated with using either anti-gp60 Ab or anti-caveolin Ab. The immunoprecipitated proteins were separated on SDS-PAGE and autoradiographed. Other details were described under “Experimental Procedures.” A, cell lysate immunoprecipitated with anti-gp60 Ab; B, cell lysate immunoprecipitated with anti-caveolin-1 Ab. Lane 1, control; lanes 2–4 were incubated for 5, 10, and 20 min, respectively. In C, BPMVEC monolayers were incubated with varying concentrations of BSA for 10 min and then the monolayers were washed and lysed. The lysates were immunoprecipitated with anti-gp60 Ab and the proteins were resolved on SDS-PAGE and transferred to nitrocellulose membrane. The membrane was immunoblotted with anti-phosphotyrosine monoclonal Ab. Lane 1, control; lanes 2–4, incubated with 0.1, 1.0, and 5.0 mg/ml BSA, respectively. In D, the same anti-phosphotyrosine blot in C was stripped and blotted with anti-gp60 Ab to indicate equal amounts of gp60 protein present. Other details were described under “Experimental Procedures.” The results are representative of three experiments.
Incubation of anti-gp60 Ab with BPMVEC at 37 °C produced dependent quantitatively on the presence of cell surface gp60. Other details are described in the legend to Fig. 2. The results are mean ± S.E. for three separate experiments carried out in triplicate. A, herbimycin A; B, genistein. The inset in B shows the effect of protein kinase C inhibitor, Calphostin C, on the uptake of albumin in BPMVEC was studied (21). *, indicates the significant difference from control ($p < 0.001$).

We also observed that albumin uptake by the cell was dependent quantitatively on the presence of cell surface gp60. Incubation of anti-gp60 Ab with BPMVEC at 37 °C produced the loss of cell surface gp60 (Fig. 4) indicative of internalization of the gp60-Ab complex (30). Uptake of albumin did not occur in these cells, indicating that cell surface gp60 expression was required for tracer albumin uptake. The time course of uptake of albumin in the present study (Fig. 2) is consistent with the kinetics of tracer gold-albumin transport across the endothelial monolayer (6–9). Uptake of $^{125}$I-albumin increased over control values within 15 min of cross-linking of gp60, but the increase was 3–4-fold within 30 min, indicating that internalization of albumin increased in a time-dependent manner. Interestingly, the amount of internalized albumin was less at 60 min; the basis of this effect is not clear, but it may be the result of exocytosis of the internalized albumin occurring within this time period (6–9). We showed that HRP (molecular mass 42 kDa) added to the incubation medium was also internalized by the endothelial cells following gp60 activation (Fig. 3). This phenomenon may be due to engulfing of fluid-phase solutes during the activation of endocytosis (35). The time course of HRP uptake was consistent with the pattern of albumin uptake with a maximum increase observed at 30 min. Potocytosis (engulfment of fluid) has also been linked to membrane budding in caveolae during the formation of endocytic vesicles (35).

The present observations are different from a recent report showing that a anti-rat gp60 Ab prevents albumin binding to rat endothelial cell and reduces pulmonary vessel wall permeability of albumin in the rat lung (36). This difference may be ascribed to several factors. First, our approach was to cross-link gp60 at 4 °C with the primary anti-gp60 Ab followed by the secondary Ab, and then to study endothelial permeability to albumin at 37 °C. We showed that gp60 cross-linking in this manner activated Src and produced gp60 clustering. Second, we prepared anti-bovine gp60 Ab using bovine pulmonary microvessel endothelial cells which are rich in vesicles (19); thus, the ability to activate albumin uptake and transport in these cells by gp60 cross-linking may be related to the relative abundance and active conformational state of the cell surface gp60. Third, the anti-gp60 Ab used in the present study may be directed against an epitope capable of activating gp60, and thereby of activating Src and the signaling machinery required for membrane budding and endocytosis.

Caveolae are non-clathrin coated pits abundant in vascular endothelial cell that have been implicated in the uptake and transcytosis of albumin across the cell layer (23, 37). Clathrin-coated pits are constitutively endocytosed, whereas caveolae remain attached to the membrane and their release from the membrane is effected by unknown mechanisms (38). Transendothelial albumin permeability was inhibited by agents that interfere with the organization of caveolae; N-ethylmaleimide (a sulfhydryl group alkylating agent) and filipin (a sterol binding drug) (37, 39) blocked albumin permeability. The caveolar membrane contains caveolin (the main scaffolding proteins of caveolae), growth factor receptors, G protein-coupled receptors, and signaling molecules such as Src family tyrosine kinases (known to phosphorylate caveolin (22, 23)) and gp60 (42). In the present study, we showed that activation with the gp60 ligand, albumin, or by cross-linking of gp60 with anti-gp60 Ab activated the Src tyrosine kinases, pp60$^c$Src and Fyn (Fig. 7). These kinases contain the Src-homology (SH2) domains which bind to phosphorylated tyrosine residues of adapter proteins, and thereby can transmit the signal to the effectors (43–47). Upon immunoprecipitating the gp60-activated cells with either anti-pp60$^c$Src Ab or anti-Fyn Ab, two additional proteins were shown to co-purify with pp60$^c$Src and Fyn (Fig. 7). The 21–23-kDa protein was identified as caveolin-1, a known substrate for Src family tyrosine kinases (22). The 36-kDa protein may be CD36 (low density lipoprotein receptor) previously shown to be associated with pp60$^c$Src and Fyn (23,
24). Gp60 activation was also associated with increased phosphorylation of caveolin-1 (Fig. 8) and enhanced binding of caveolin-1 with pp60^Src and Fyn (Fig. 7). Src kinases have been shown to phosphorylate caveolin at the amino-terminal tyrosine residues (22). Caveolin also exists as homoligomer which has the capacity to self-associate into larger complexes (22). These tyrosine-phosphorylated caveolin may serve as a docking site for SH2 domain signaling molecules (22, 43–47). Thus, the phosphorylation of gp60 as well as caveolin-1 and the upstream Src kinases induced by gp60 activation may be important in activating the PTK signaling pathway required for albumin endocytosis and transcytosis.

To address the mechanism of the albumin endocytosis and transcytosis induced by gp60 activation, we studied the function of the Src PTK signaling pathway in mediating albumin endocytosis following gp60 activation. We determined the effects of herbimycin A and genistein (inhibitors of the tyrosine kinase pathway (48, 49)). Both agents prevented in a dose-dependent manner the increase in albumin uptake and permeability in control and endothelial cells activated with anti-gp60 Ab (Fig. 9). The protein kinase C inhibitor (calphostin C), in contrast, had no such effect. These findings indicate the importance of the PTK pathway in regulating the gp60-activated albumin endocytosis and transcytosis.

In summary, the results indicate that gp60 activation by cross-linking or albumin induces the activation of Src kinases and tyrosine phosphorylation of gp60 and caveolin-1 in the endothelial cell monolayer. The release of caveolae from the endothelial plasma membrane and endocytosis following gp60 activation involves the activation of the PTK signaling pathway. Tyrosine phosphorylation of gp60 may recruit SH2-domain proteins such as Src kinases that then induce phosphorylation of caveolin-1. Therefore, the activation of caveolin-1 and gp60 by Src kinases may be critical signaling events responsible for initiating albumin endocytosis and transcytosis across endothelial cells.

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