Endothelial Cell-surface gp60 Activates Vesicle Formation and Trafficking via G\textsubscript{i}-coupled Src Kinase Signaling Pathway

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Abstract. We tested the hypothesis that the albumin-docking protein gp60, which is localized in caveolae, couples to the heterotrimeric GTP binding protein G\textsubscript{i}, and thereby activates plasmalemmal vesicle formation and the directed migration of vesicles in endothelial cells (ECs). We used the water-soluble styryl pyridinium dye N-(3-triethylaminopropyl)-4-(p-dibutylaminostyryl) pyridinium dibromide (FM 1-43) to quantify vesicle trafficking by confocal and digital fluorescence microscopy. FM 1-43 and fluorescently labeled anti-gp60 antibody (Ab) were colocalized in endocytic vesicles within 5 min of gp60 activation. Vesicles migrated to the basolateral surface where they released FM 1-43, the fluid phase styryl probe. FM 1-43 fluorescence disappeared from the basolateral EC surface without the loss of anti-gp60 Ab fluorescence. Activation of cell-surface gp60 by cross-linking (using anti-gp60 Ab and secondary Ab) in EC grown on microporous filters increased transendothelial \textsuperscript{125}I-albumin permeability without altering liquid permeability (hydraulic conductivity), thus, indicating the dissociation of hydraulic conductivity from the albumin permeability pathway. The findings that the sterol-binding agent, filipin, prevented gp60-activated vesicle formation and that caveolin-1 and gp60 were colocalized in vesicles suggest the caveolar origin of endocytic vesicles. Pertussis toxin pretreatment and expression of the dominant negative construct encoding an 11–amino acid G\textsubscript{i} carboxyl-terminal peptide inhibited endothelial \textsuperscript{125}I-albumin endocytosis and vesicle formation induced by gp60 activation. Expression of dominant negative Src (dn-Src) and overexpression of wild-type caveolin-1 also prevented gp60-activated endocytosis. Caveolin-1 overexpression resulted in the sequestration of G\textsubscript{i} with the caveolin-1, whereas dn-Src inhibited G\textsubscript{i} binding to caveolin-1. Thus, vesicle formation induced by gp60 and migration of vesicles to the basolateral membrane requires the interaction of gp60 with caveolin-1, followed by the activation of the downstream G\textsubscript{i}-coupled Src kinase signaling pathway.

Key words: transcytosis • endocytosis • caveolae • microvascular endothelial cells • albumin permeability

Introduction

Albumin is the primary plasma protein that maintains the colloid osmotic pressure gradient across the semi-permeable microvascular endothelial barrier and, hence, the plasma interstitial albumin concentration gradient is critical in regulating tissue fluid balance (for review see Lum and Malik, 1994). A transcellular pathway through endothelial cells has been implicated in the transport of albumin (Ghitescu et al., 1986; Milič et al., 1987; Shasby and Peterson, 1987; Michel, 1992), lipids, hormones, peptides, and drugs that bind avidly to albumin (Peters, 1975; Partridge, 1979; Forker and Luxton, 1983). The endothelial cell surface 60-kD glycoprotein, gp60\textsuperscript{1} or albondin (Schnitzer et al., 1988; Ghinea et al., 1989; Schnitzer and Oh, 1994; Tiruppathi et al., 1996), is believed to be important in the mechanism of albumin binding and activation of transcellular albumin transport via vesicles. A albumin can also cross the endothelial barrier via a diffusional-convective paracellular pathway (Lum and Malik, 1994). Inflammatory

\textsuperscript{1}Abbreviations used in this paper: Ab, antibody; BLMVEC, bovine lung microvessel endothelial cells; cav-1, caveolin-1; DA PI, 4',6-diamidino-2-phenylindole, dihydrochloride; dn-Src, dominant negative Src; EC, endothelial cell; FM 1-43, N-(3-triethylaminopropyl)-4-(p-dibutylaminostyryl) pyridinium dibromide; GFP, green fluorescent protein; gp60, 60-kD albumin-docking protein; \textit{L}\textsubscript{H}, hydraulic conductivity; LSM, laser scanning microscope; RH 414, N-(3-triethylaminiumpropyl)-4-(4-(diethylamino)phenyl)butadienyl)pyridinium dibromide; RT-PCR, reverse transcription-PCR.
mediators such as thrombin and histamine increase endothelial albumin permeability by increasing the size of interendothelial clefts (Majno et al., 1969; Garcia et al., 1986; Del Vecchio et al., 1987; Qiao et al. 1995; Rabiet et al., 1996).

We purified gp60 from pulmonary microvascular endothelial cells and showed it could bind specifically and in a saturable manner to albumin (Tiruppathi et al., 1996). We also showed that activation of the cell-surface gp60 using a cross-linking antibody (Ab) induced the transendothelial flux of albumin (Tiruppathi et al., 1997). Inhibitor studies suggested the increased albumin flux involved the activation of Src family tyrosine kinases that phosphorylated caveolin-1 and gp60 (Tiruppathi et al., 1997). Formation of vesicles induced by gp60 was inhibited by the tyrosine kinase inhibitors, herbimycin A and genistein (Tiruppathi et al., 1997; Niles and Malik, 1999). Trafficking of vesicles to the basolateral membrane in endothelial cells also required the N-ethylmaleimide–sensitive fusion factor (NSF) and soluble NSF attachment protein receptor (N-N and M-alk, 1999).

The heterotrimeric GTP binding protein, Gαi, binds to caveolin-1 in the caveolar membrane (Li et al., 1995; Okamoto et al., 1998) and activates Src kinases (Uttrell et al., 1996; Ighisi and Gukkind, 1998; Eilis et al., 1999), which also bind to caveolin-1 (Li et al., 1996). Since gp60 is localized in the caveolar membrane (Schnitzer and Oh, 1994) and is capable of activating Src family tyrosine kinases (Tiruppathi et al., 1997), we addressed the possible role of the Gαi-coupled Src kinase pathway in gp60-activated signaling vesicle formation and trafficking in endothelial cells.

**Materials and Methods**

**Antibodies**

gp60 Ab was prepared as described previously (Tiruppathi et al., 1996). Polyclonal anti-gp60 Ab was labeled with the Cy3 bisfunctional reactive dye (Tiruppathi et al., 1997). Monoclonal and polyclonal anti–caveolin-1 antibodies were obtained from Transduction Laboratories. Rabbit-anti-Gαi polyclonal Ab recognizing the carboxyl-terminal region of isoforms 1 and 2 (KHNLDQGLFL) was purchased from Calbiochem. Goat anti–rabbit and anti–mouse IgG labeled with rhodamine, FITC, Alexa 568, or Alexa 488 and BSA-Alexa 488 conjugate were purchased from Kirkegaard & Perry Laboratories and Molecular Probes, Inc.

**Fluorescent Probes**

We used fluorescent water-soluble styryl pyridinium dyes N-(3-triethylamino(propyl))-4-(p-dibutylaminostyryl) pyridinium dibromide (FM 1-43) and N-(3-triethylammoniumpropyl)-4-(4-(4-diethylaminophenyl) butadienyl) pyridinium dibromide (R 414; Molecular Probes, Inc.) to label plasmalemma-derived vesicles (Niles and Malik, 1999). Stock solutions of 5 mg/ml were prepared in DMSO and stored in a desiccator at ~80°C for up to 1 mo. Cell staining solutions (5 μg/ml) were made in H BSS containing 20 mM Hepes, 2 mM CaCl2, and 2 mM MgCl2. Staining solutions and subsequent washing buffer contained fixed BSA concentration (6 mg/ml; fraction V, 99% pure, endotoxin-free; Sigma Chemical Co.).

**Albumin Iodination**

BSA was labeled with Na125I (New England Nuclear) using the chloramine T procedure (Bocci, 1964). Free iodine-125, separated from 125I-albumin with a Sephadex G 25 column, constituted <0.3% of the total radioactivity.

**Endothelial Cell (EC) Cultures**

Bovine lung microvessel ECs (BLMVEC) were isolated and cultured in high glucose DME (GIBCO BRL) supplemented with 10% FBS (HyClone), 5 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Del Vecchio et al., 1992). Endothelial cell cultures were maintained in an incubator at 37°C in 5% CO2/95% room air.

**Plasmid DNA Preparations**

Wild-type (wt) caveolin-1 was prepared from human umbilical vein ECs endothelial cell by reverse transcription-PCR (RT-PCR). The sequenced RT-PCR product was identical to human caveolin-1. The RT-PCR product was subcloned into pcDNA 3.1 (Invitrogen) and used for transfection studies. D ominantly negative S (dn-S) (Y527F, K295M) and wt-Src in vector p5M were obtained from Dr. Silvio Gutkind (National Institute of Dental Research, National Institutes of Health, Bethesda, MD). Gαi and Gαs dominantly negative minigene encoding the 11-amino acid carboxyl terminus of GαR (IKNNLDQGLFL) or Gαq (QLYNKLKEYNAV; Gilchrist et al., 1998) were ligated into pCDNA 3.1 plasmid vector. A minigene encoding a scrambled sequence of Gαq (NGIKCLSNKD) was used as a negative control. Green fluorescent protein (GFP) plasmid DNA (plasmid pGREEN LAN-TE R N-1) was purchased from Gibco BRL.

125I-Albumin Endocytosis

Endocytosis of 125I-albumin was measured as described previously (Tiruppathi et al., 1997). BLMVEC were grown to confluence in either 6- or 12-well culture plates. Monolayers were washed twice with 10 mM Hepes-buffered DME, pH 7.4, and the uptake of 125I-albumin was carried out for 25 min at 37°C. Endocytosis was terminated by placing EC monolayers on ice and washing three times with either pH 2.5 buffer (0.2 M acetic acid and 0.5 M NaCl) or pH 3.0 buffer (20 mM glycine-HCl and 0.15 M NaCl) to remove the surface-bound 125I-albumin (Tiruppathi et al., 1992). Cells were lysed with 50 mM Tris-HCl buffer, pH 7.4, containing 1% Triton X-100 and 0.5% SDS, and the lysate was used to measure endocytosis of 125I-albumin.

**Transendothelial 125I-Albumin Permeability**

Transendothelial 125I-BSA permeability of BLMVEC monolayers grown on microporous polycarbonate transwell filter inserts (Corning Costar Corp.) was measured as previously described (Del Vecchio et al., 1987; Siffinger-Birnboim et al., 1988).

**Endothelial Monolayer Hydraulic Conductivity (Lp)**

Confluent BLMVEC on gelatin-coated microporous polycarbonate filters were used to measure transendothelial fluid flux using the two-compartment system (Qiao et al., 1993). Both upper and lower chambers were filled with 0.5% albumin in HBSS, pH 7.4, at 37°C and sealed with rubber stoppers. Constant flow perfusion was used to renew the luminal fluid every minute and to prevent an increase in osmolarity. Hydrostatic pressure in the upper chamber was controlled by adjusting the height of the outflow tubing. The filtration rate was collected in 1-min intervals and the filtration rate per unit surface (Jv) was calculated as the timed movement of fluid meniscus in the collecting tubing. The filtration rate at the two given pressures (P1 = 0 cm H2O and P2 = 5 cm H2O) at 20-min intervals was measured for up to 2 h. The Lp was calculated as (Jv1 – Jv2) / (P1 – P2) (Qiao et al., 1993).

**Cell-surface gp60 Cross-linking**

BLMVEC monolayers were washed twice with Hepes-buffered DME (at 4°C) and incubated with 10 μg/ml of anti-gp60 Ab, followed by 10 μg/ml of a secondary Ab (goat anti-rabbit) for 30 min at 4°C (Tiruppathi et al., 1997). A fiter treatment, the cells were rewarmed to 37°C for the experiments.

**Styryl Pyridinium Dye Uptake**

Endocytosis in endothelial cells was quantified as described previously (Niles and Malik, 1999) using the styryl pyridinium dyes, FM 1-43 and R 414. In brief, 5 × 104 BLMVEC were plated on 23-mm-diam N. 1 coverslips or a Lab-tek chamber slides (Nalge Nunc Inc.) coated with 0.1% gelatin. Cells were incubated in buffer containing 5 μg/ml FM 1-43 or R 414 at 37°C for 15 min, and then washed three times in ice-cold HBSS. A styryl pyridinium dyes fluoresce brightly at the membrane-water interface (Bet et al., 1992). FM 1-43 fluorescence associated with the cells after washing cell surface probe was the result of incorporation of FM 1-43 into plasmalemma-derived vesicles.
Digital Fluorescent Microscopy

Live cell fluorescent imaging was performed with an inverted Nikon microscope as previously described (Niles and Malik, 1998). Fluorescence and differential interference contrast images were recorded for each cell field with a cooled integrating charge-coupled device camera (Imagepoint; Photometrics). Quantitative analysis of images (median cell brightness and number of particles per cell) was performed using Image Pro Plus software with custom written functions as previously described (Niles and Malik, 1999). Data expressed as median cell brightness or number of particles per cell were consistent within each treatment.

Colocalization of gp60 and FM 1-43

Confluent BLMVEC on coverslips were incubated for 15 min at 37°C in a mixture of 5 μg/ml FM 1-43 and 5 μg/ml Cy3-conjugated anti-gp60 A b in HBSS plus 10 mg/ml BSA to colabel cytosolic vesicles. Cells were washed quickly three times with ice-cold HBSS containing 10 mg/ml albumin to remove external FM 1-43 and three times for 5 min each with ice-cold pH 5.0 buffer (0.1 M NaCl plus 0.05 M sodium acetate) to remove Cy3-labeled anti-gp60 A b attached to cell-surface gp60. The cells were warmed to 37°C for 5 or 45 min to allow the colabeled vesicles to migrate. Cells were viewed by laser scanning confocal microscopy (laser scanning microscope [LSM] 410 and 510; Carl Zeiss) in sequential optical sections. The section plane was advanced in 0.1-μm increments through the cell from the apical to the basolateral side. A 1.4 mm optical plane, the specimen was scanned at 488 nm to excite FM 1-43 and then at 568 nm to excite Cy3 (argon/Acrytophan laser). FM 1-43 (green) and Cy3 (red) images were overlaid and analyzed for coincident red and green pixels (thus the colocalized fluorescence was in yellow).

Localization of gp60, Giai, and Caveolin-1

Cellular localization of Giai, gp60, and caveolin-1 in the plasma membrane and plasmalemmal vesicles of endothelial cells exposed to albumin or gp60 cross-linking (described above) was determined by immunocytochemical labeling and laser scanning confocal microscopy (Zeiss LSM 210 and 510). BLMVEC were serum-deprived for 24 h, washed three times with Hepes-buffered HBSS or phenol red-free DMEM, and exposed to 6 mg/ml BSA in the presence or absence of 50 μg/ml Alexa 488-conjugated BSA and/or 3.5 μg/ml Cy3-anti-gp60 A b for up to 30 min. Cells were either washed three times with HBSS and imaged live, or fixed with 4% paraformaldehyde in HBSS and blocked for 30 min in HBSS containing 5% goat serum, 0.1% Triton X-100, and 0.01% NaN3. Primary Ab labeling was performed overnight at 4°C with anti-caveolin-1 mA b (1:1 μg/ml), polyclonal anti-Gi A b (1:100 dilution), or 20 μg/ml anti-gp60 IgG. Coverslips were washed three times for 10 min in HBSS, blocked for 30 min with 5% goat serum, and incubated for 2 h at room temperature with fluorescently labeled goat anti-rabbit and goat anti-mouse Ab. In some cases, 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; 1 μg/ml) was added to visualize the nucleus. Confocal microscopy was performed using 364-, 488-, and 568-nm excitation laser lines to detect DAPI (BP385-470 nm emission), FITC/Alexa 488 (BP505-550 nm emission), and rhodamine/Alexa 568 fluorescence (LP585 emission) in optical sections <1 μm in thickness (pinhole set to achieve 3 Airy unit).

Pertussis Toxin Treatment

Confluent BLMVEC were incubated in DMEM containing 100 ng/ml pertussis toxin for 6 h at 37°C. Control cells were incubated in toxin-free medium for 6 h before labeling with the styryl dye.

Filipin Treatment

Confluent BLMVEC were serum-deprived for 24 h and pretreated with 50 nM filipin (25 ng/ml) for 30 min at 37°C. Control cells were incubated with serum- and phenol red-free DMEM containing vehicle DMSO (0.005% DMSO).

Plasmid Transfection Studies

Expression of Giai minigene, wt-caveolin-1, and dn-Src. BLMVEC were grown to 50% confluence in 60-mm-diam plates and transfected using Ef...
fectene (QIAGEN Inc.) according to manufacturer’s protocol. Plasmid DNA–Effectene complexes containing either 0.25 μg/ml vector alone, wt-caveolin-1, wt-Src, dn-Src, dn-PKA, or Gai-, Gag-, or Gai-random sequence minigene constructs were incubated with 0.25 μg/ml GFP (GIBCO BRL) in DME containing 10% FBS. The transfection mixture was removed after 6 h and fresh media containing 10% FBS was applied. At 24 h after transfection, cells were trypsinized and transferred to either multiwell Lab-tek chambers for styryl pyridinium dye uptake studies, to 35-mm-diam wells for obtaining cell lysates, or to 12 mm-diam No. 1 glass coverslips for immunostaining. Cells were allowed to grow for an additional 24 h, and then used for experiments (48 h after transfection). Transfection efficiency was >30%.

**Coimmunoprecipitation of Caveolin-1 and gp60**

Confluent BLMVEC in 60-mm-diam culture dishes were kept in serum-free medium overnight and metabolically labeled with 200 μCi/ml 32P-orthophosphate for 4 h. Cells were stimulated for 20 min with 6 mg/ml BSA and lysed (30 min at 4°C in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1.0% NP-40, 0.1% SDS, 1 mM Na3VO4, 1 mM NaF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 44 μg/ml PMSF). Insoluble material was removed by centrifugation (13,000 g for 15 min) before overnight immunoprecipitation with preimmune IgG (10 μg/ml), anti-gp60 Ab (10 μg/ml), or polyclonal anti–caveolin-1 Ab (1 μg/ml) at 4°C. Protein A or G agarose beads were added to each sample and incubated for 1 h at 4°C. Immuneprinci-
were used for transendothelial 125I-albumin permeability measurements at 37°C. Both luminal and albuminal compartments contained 30 mg/ml of unlabeled albumin. A sterisk indicates the difference from control (P < 0.001).

(b) Lack of effect of gp60 cross-linking on endothelial monolayer hydraulic conductivity (Lp). A system consisting of BLMVEC cultured on filters using the two compartment system was used to measure Lp (Qiao et al., 1993); medium albumin concentration was 5 mg/ml. Monolayers were used for transendothelial 125I-albumin permeability measurements at 37°C. Both luminal and albuminal compartments contained 30 mg/ml of unlabeled albumin. A sterisk indicates the difference from control (P < 0.001).

To determine the effects of gp60 cross-linking on vesicle formation, BLMVEC were incubated with either anti-gp60 A b or preimmune IgG (control A b) for 30 min at 4°C followed by anti-rabbit secondary Ab for 30 min. Cells were exposed to FM 1-43 for 15 min at 37°C, membrane-bound dye was washed, and intracellular FM 1-43 fluorescence was quantified as median brightness of cells from the apical to basolateral cell membrane. Colocalization was determined by merging red (Cy3 fluorescence) and green images (Fig. 3 a; FM 1-43 fluorescence). Although FM 1-43 labels all plasmalemmal-derived vesicles, a fraction of these vesicles were colocalized (yellow) with the fluorescently labeled gp60 A b.

Activation of gp60 Induces Plasmalemmal Vesicle Formation

The cellular distribution of gp60 was determined after incubating confluent endothelial cells for 30 min with Cy3-conjugated anti-gp60 A b. We observed punctate distribution of fluorescence after incubation of the probe at 37°C, followed by cold (4°C) wash buffer to remove the cell surface-bound Cy3 probe (Fig. 1 a). To visualize internalized gp60, cell-surface fluorescence was removed by an acid wash. The resultant image showed that gp60 was internalized during the incubation period (Fig. 1 b). Reduction of the temperature to 4°C followed by a low pH wash prevented the gp60 internalization (Fig. 1 c).

Endothelial cell monolayers were incubated with steryl pyridinium dye, FM 1-43 (5 μg/ml), for 15 min at 37°C, and then rinsed with dye-free buffer to visualize plasmalemma-derived endocytotic vesicles. Endothelial cells incubated in FM 1-43 exhibited the punctate distribution of fluorescence characteristic of vesicles (Fig. 1 d). Vesicle formation was inhibited when cells were kept at 4°C during the dye loading period (Fig. 1 e).

To determine the fate of gp60 and of vesicles formed by gp60 activation, FM 1-43-labeled vesicles were colocalized with the Cy3-conjugated anti-gp60 A b. Cells were washed with acid buffer to detach Cy3-anti-gp60 A b from the cell surface before imaging. Confocal images of each fluorescent probe were obtained at 0.1-μm z-axis step increments from the apical to basolateral cell membrane. Colocalization was determined by merging red (Cy3 fluorescence) and green images (Fig. 3 a; FM 1-43 fluorescence). Although FM 1-43 labels all plasmalemmal-derived vesicles, a fraction of these vesicles were colocalized (yellow) with the fluorescently labeled gp60 A b.

Activation of gp60 Induces Transcellular Migration of Plasmalemma-derived Vesicles

To determine the fate of gp60 and of vesicles formed by gp60 activation, FM 1-43-labeled vesicles were colocalized with the Cy3-conjugated anti-gp60 A b. Cells were washed with acid buffer to detach Cy3-anti-gp60 A b from the cell surface before imaging. Confocal images of each fluorescent probe were obtained at 0.1-μm z-axis step increments from the apical to basolateral cell membrane. Colocalization was determined by merging red (Cy3 fluorescence) and green images (Fig. 3 a; FM 1-43 fluorescence). Although FM 1-43 labels all plasmalemmal-derived vesicles, a fraction of these vesicles were colocalized (yellow) with the fluorescently labeled gp60 A b.

To follow the migration of plasmalemma-derived vesicles, endothelial cells were colabeled with fluorescent anti-gp60 A b and FM 1-43 for 15 min at 37°C, washed (4°C), and rewarmed (37°C) for either 5 or 45 min to activate the vesicles.

Western Blot Analysis
BLMVEC lysates (10 μg protein was loaded per lane) were resolved by SDS-PAGE on a 12% separating gel under reducing conditions and transferred to Duralose membrane. Membranes were blocked with 5% dry milk in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20 for 2 h at 22°C. Membranes were incubated with a 1:3,000 dilution of polyclonal anti-caveolin-1 A b or 0.4 μg/ml anti-Gi or Goeta A b at 22°C for 3 h. After washing, membranes were incubated at 22°C with HRP-conjugated goat anti-rabbit Ab. After incubation, membranes were washed twice, and protein bands were localized by incubating with enhanced chemiluminescence reagent (Pierce Chemical Co.).

Statistical Analysis
Statistical comparisons were made using the t test with the significance level set at P < 0.05.
icle migration. At 5 min after colabeling, the vesicles that formed during the dye incubation period were located in the cytosol near the apical plasmalemma; i.e., in upper one-third of each cell (Fig. 3 a, top row). At 45 min after colabeling, the vesicles containing gp60 and endocytic marker dye had migrated to the lower half of the cell near the basolateral plasmalemma (Fig. 3 a, bottom row).

Fig. 3 b shows the colocalization of fluorescence in different optical section depths of the cell at either 5 or 45 min after gp60 activation. At 5 min after gp60 activation, the peak colocalized fluorescence (i.e., the gp60-activated vesicles) was observed <0.5 µm from the apical surface. At 45 min, the peak colocalized fluorescence was shifted toward the basolateral cell surface (i.e., at a section depth of 1.5 µm). Because of the release of styril dye into the basolateral extracellular compartment, the immunofluorescence intensity of the 45-min peak was markedly diminished, such that residual immunofluorescence primarily reflected the persistence of gp60 antigen in the basolateral aspect of the cell.

**Effects of gp60 Activation on Transendothelial 125I-Albumin Flux and Endothelial Hydraulic Conductivity**

We measured transendothelial flux of tracer 125I-albumin and hydraulic conductivity in confluent BLMVEC monolayers grown on polycarbonate filters to determine gp60-activated transendothelial albumin permeability and its relationship to liquid permeability. Cross-linking of gp60 at 37°C using 10 µg/ml anti-gp60 Ab plus secondary Ab increased transendothelial 125I-albumin permeability by twofold (Fig. 4 a). However, the increased 125I-albumin permeability was dissociated from endothelial monolayer hydraulic conductivity (Lp, Fig. 4 b). As gp60 activation increased transendothelial albumin permeability without increasing water permeability, the results indicate increased albumin flux through transcellular pathways.

**Colocalization of gp60 and Albumin in Plasmalemma-derived Vesicles**

To determine the pattern of localization of gp60 and albu-
After activation of gp60, we evaluated immunostaining of cells after a 30 min incubation (37°C) with Cy3-anti-gp60 Ab and Alexa 488 BSA in phenol red-free DME containing 5 mg/ml albumin. Fig. 5a shows the punctate fluorescent structures containing gp60 Ab (red), DAPI staining of the nucleus (blue), and fluorescently labeled albumin (green) near the apical membrane of BLMVEC. An overlay image (Fig. 5a, far right) of the three probes shows a high degree of gp60 and albumin colocalization (in yellow). A projection image of BLMVEC gp60, DAPI, and
Figure 7. Role of $G_i$ signaling in gp60-induced vesicle formation. (a) Cell-surface $G_{ai}$ immunostaining decreases after gp60 activation. BLMVEC exposed to vehicle (left) or anti-gp60 Ab (right) for 10 min at 37°C were labeled with polyclonal anti-$G_{ai}$ Ab plus Alexa 488-conjugated goat anti-rabbit secondary Ab. Confocal images (eight frame average, <1.0-µm thick optical sections near the apical plasma membrane) were acquired with a Zeiss LSM 210. (b) Pertussis toxin inhibits gp60-activated vesicle formation. BLMVEC were preincubated for 6 h at 37°C in medium containing 100 ng/ml pertussis toxin. At end of the preincubation period, endocytosis was stimulated by 30 min of incubation in 5 µg/ml anti-gp60 Ab to cross-link gp60. FM 1-43 (5 µg/ml) was added to cells during the final 15 min of Ab incubation; excess styryl dye was washed away with three changes of ice-cold buffer. Control cells received no toxin during 6-h preincubation period. Median cellular fluorescence intensity is shown. Pertussis toxin blocked FM 1-43 endocytic marker dye uptake that was induced by the activation of gp60 with anti-gp60 Ab (gp60 cross-linking). The asterisk indicates an increase ($P < 0.05$) of median fluorescence intensity because of gp60 cross-linking (in the absence of pertussis toxin). (b, inset) Fluorescent images of cells: (left) no pertussis toxin; (right) pertussis toxin treatment. Results are representative of three experiments. (c) Pertussis toxin (100 ng/ml for 6 h) inhibits $^{125}$I-albumin uptake in endothelial cells induced by gp60 cross-linking. Cells were incubated in $^{125}$I-labeled tracer albumin for 25 min after gp60 cross-linking, washed three times in pH 2.5 buffer, lysed, and counted for $^{125}$I-labeled albumin. Bars indicate SD ($n = 3$). The single asterisk indicates a difference from no anti-gp60 Ab (−) control group ($P < 0.05$); and the double asterisk indicates a decrease relative to the anti-gp60 Ab-activated
albumin fluorescent staining is shown in Fig. 5 b. The en face (x-y) view of stacked z-plane images (22 sections) showed marked colocalization of gp60 A b and albumin in the same endocytic vesicles (Fig. 5 b).

**Gp60 and Caveolin-1 Interaction**

We metabolically labeled serum-deprived BLMVEC with \(^{32}P\)-orthophosphate for 4 h, stimulated with or without 6 mg/ml albumin for 20 min, and prepared the cell lysates for immunoprecipitation to study caveolin-1 and gp60 interactions (see M and Methods). Incubation of the cell lysate with control Ab failed to precipitate either gp60 or caveolin-1 (Fig. 6 a, lanes 1 and 4). In the absence of albumin, anti-gp60 Ab or anti-caveolin-1 Ab immunoprecipitates revealed 60- and 22-kD proteins (Fig. 6 a, lane 2 and 3), which corresponded to gp60 and caveolin-1, respectively. However, in the case of albumin stimulation, anti-gp60 A b immunoprecipitated 60-, 36-, and 22-kD proteins (Fig. 6 b, lane 5). The 60- and 22-kD bands were identified as gp60 and caveolin-1, respectively, by Western blotting (not shown). The 36-kD band may be CD36, the low density lipoprotein receptor (Huang et al., 1991; Lisanti et al., 1994; Tiruppathi et al., 1997). In addition, anti-caveolin-1 A b precipitated caveolin-1 as well as gp60 in the albumin-stimulated cells (Fig. 6 b, lane 6). Thus, the physical association of gp60 with caveolin-1 in endothelial cells is facilitated by the activation of gp60 with albumin.

Serum-deprived BLMVEC were stimulated with albumin for 0, 3, or 30 min, and were fixed and stained with anti-gp60 and caveolin-1 A bs to address whether gp60 and caveolin-1 immunostaining comigrated from the apical to basolateral surfaces as a function of albumin exposure. In the absence of albumin, gp60 (red) and caveolin-1 (green) immunostaining, which showed a marked overlap (in yellow), appeared near the apical surface of BLMVEC monolayers (Fig. 6 c), as seen in the z-axis optical sections (top left, apical surface; bottom right, basolateral surface). Exposing cells to 6 mg/ml BSA for 3 min (Fig. 6 d) or 30 min (Fig. 6 e) to activate gp60 induced the internalization and basolateral migration of gp60. The green fluorescence, which marked caveolin-1, did not redistribute towards the basolateral membrane to the same extent as gp60. In control experiments, non-specific immunofluorescence observed in the presence of preimmune rabbit IgG, control mouse IgG, or with secondary antibodies alone was minimal; it did not show a pattern of colocalization and also did not change after the exposure of endothelial cell monolayers to albumin.

BLMVEC were treated with filipin to determine whether disruption of caveolae influenced albumin uptake induced by gp60. BLMVEC were incubated in the presence or absence of 50 nM filipin for 30 min at 37°C and incubated with anti-gp60 A b for 30 min at 4°C to cross-link gp60. Cells were incubated with Alexa 488-BSA for 30 min in media containing 5 mg/ml of unlabeled BSA and acid-washed to remove extracellular label. As shown in Fig. 6 g, filipin prevented the formation of vesicles as compared with control cells (Fig. 6 f).

**G, Transduces gp60-activated Vesicle Formation**

We used three approaches to address the role of G in the mechanism of gp60-induced vesicle formation and trafficking: (1) pertussis toxin, which prevents activation of G\(_{i}\)/G\(_{0}\) by ADP ribosylation (Eppler and Gilman, 1992); (2) transfection of the dn construct encoding the 11–amino acid carboxyl-terminal peptide sequence of G\(_{ai122}\); which competes for G\(_{ai}\)–receptor interactions (Gilchrist et al., 1998); and (3) G\(_{ai}\) immunostaining.

In control BLMVEC, G\(_{ai}\) was localized in the apical membrane (Fig. 7 a, left). Cross-linking of gp60 for 10 min resulted in a decreased apical membrane–associated G\(_{ai}\) immunostaining (Fig. 7 a, right). Pretreatment of endothelial monolayers with the pertussis toxin prevented both FM 1-43 and \(^{125}\)I-albumin endocytosis induced by gp60 (Fig. 7 b and c). In addition, the expression of G\(_{ai}\) carboxyl-terminal peptide (dn-G\(_{ai}\)) in BLMVEC also prevented the gp60-activated vesicle formation as determined by RH 414 uptake in green fluorescent protein (GFP)–positive endothelial cells (Fig. 7 d and e). In control experiments, endothelial cells transfected with the G\(_{ai}\) minigene (dn-G\(_{ai}\)) or control minigene encoding a randomized sequence of G\(_{ai}\) carboxy terminus (G\(_{ai}\)-random) showed the characteristic activation of endocytosis secondary to gp60 stimulation.

**Expression of Caveolin-1 and dn-Src Inhibit Vesicle Formation**

We studied the effects of overexpression of wt-caveolin-1 since caveolin-1 can bind to G\(_{ai}\) (Li et al., 1995) and, thus, may functionally inactivate endogenous G\(_{ai}\). Since caveolin-1 and gp60 are phosphorylated by Src family kinases after gp60 activation (Tiruppathi et al., 1997), we also studied the role of Src by inducing the expression of dn-Src. Whole cell lysates from BLMVEC transfected with vector alone (Mock), wt-caveolin-1 (wt-Cav-1), or dn-Src were immunoprecipitated with anti–caveolin-1 A b, separated by SDS-PAGE, and blotted with A bs against caveolin-1, G\(_{ai}\), and G\(_{ai}\)-random (Fig. 8). The resulting blots showed a twofold increase in expression of caveolin-1 in cells transfected with wt-caveolin-1 (lane 2) compared with mock-controls.
The present study demonstrates an important role of gp60 in stimulating endocytosis and the directed migration of vesicles in endothelial cells. A nalysis of serial confocal sections showed the apical-to-basolateral migration of vesicles activated by gp60 in live endothelial cells, which is suggestive of a transcytotic process. We showed that vesicular markers (FM 1-43 and RH 414) were taken up in the apical membrane-derived vesicles and that the targeting of vesicles was observed at the basolateral endothelial cell surface. Interestingly, gp60 remained localized in the basolateral vesicles after the release of styryl dye, suggesting that gp60 has the potential to recycle to the apical membrane and to reactivate endocytosis. In addition, we showed that gp60 activation (with cross-linking A b) also increased 125I-labeled albumin uptake across endothelial monolayers and the transcellular migration of steryl pyridinium dye-filled vesicles. Thus, the activation of gp60-induced transcellular membrane traffic was associated with increased transendothelial albumin permeability. However, gp60 activation did not change the endothelial barrier Lp, suggesting that the interendothelial junctional or paracellular permeability pathway did not increase when albumin transport was stimulated by gp60. These physiological experiments provide further proof that increased albumin permeability after gp60 activation occurred via a transcellular or nonhydrostatic permeability pathway.

Caveolae, the nonclathrin-coated pits that are abundant in vascular endothelial cells, have been implicated in the mechanism of endocytosis (Lisanti et al., 1994; A derson, 1998) and transcellular permeability (Milici et al., 1987; Schnitzer et al., 1994; Tiruppathi et al., 1997). We showed that gp60 and caveolin-1, the caveolar structural protein
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(Rothberg et al., 1992), were colocalized in endothelial cell apical plasma membrane-derived vesicles, and that they migrated in the basolateral direction after activation of gp60. However, caveolin-1 did not redistribute towards the basolateral membrane to the same extent as gp60, suggesting that gp60 translocates across the cell mainly, though not exclusively, with caveolin-1. Immunoprecipitation data showed that gp60 and caveolin-1 were physically associated after gp60 activation, which is suggestive of a critical interaction between gp60 and caveolin-1. Endocytosis of fluorescent albumin was blocked by filipin, a sterol-binding agent that disassembles the cholesterol-rich caveolae (Rothberg et al., 1990, 1992; Schnitzer et al., 1994). Taken together, these data support the concept that gp60-activated albumin endocytosis occurs by means of vesicles derived from caveolae. The budding of vesicles may be the result of dynamin-regulated fission of the caveolar plasmalemmal membrane (Oh et al., 1998).

Plasmalemmal membrane-derived vesicles have been shown to contain caveolin-1, G proteins, G protein-coupled receptors, and Src family tyrosine kinases (Chun et al., 1994; Li et al., 1996; Liu et al., 1996; Murphy et al., 1996; Song et al., 1997; Zacchi et al., 1998; for review see Anderson, 1998). Caveolin-1 is known to sequester caveolae-associated signaling proteins such as Src and G α in their inactive form (Okamoto et al., 1998). As caveolin-1 may be involved in gp60-induced vesicle formation (Milici et al., 1987; Tiruppathi et al., 1997), we overexpressed caveolin-1...
in endothelial cells and addressed the effects of this interaction on vesicle formation after gp60 activation. We observed that caveolin-1 overexpression sequestered G_{i} and, importantly, it prevented gp60-activated vesicle formation. This finding is consistent with the role of caveolin-1 in binding (and thereby in sequestering) G proteins and other signaling molecules (Okamoto et al., 1998). We addressed the possible role of G_{i} in activating gp60-induced vesicle formation using pertussis toxin (Hepler and Gilman, 1992) and the expression of G_{i} antagonist peptide (Gilchrist et al., 1998; Eiils et al., 1999). A both inhibitors prevented gp60-activated vesicle formation in endothelial cells, the results indicate that G_{i} plays a critical role in transducing the gp60-induced vesicle formation.

Since G_{i} induces the activation of downstream Src kinase (Luttrell et al., 1996; Igishi and Gutkind, 1998), we addressed whether Src kinase was also involved in the gp60-activated signaling cascade. The results indicated that the expression of dn-Src prevented gp60-activated vesicle formation in endothelial cells. Interestingly, dn-Src expression also prevented the binding of G_{i} to caveolin-1. A Src kinase and G_{i} can compete for a common binding domain on caveolin-1 (Li et al., 1996; Okamoto et al., 1998), an explanation for our finding is that binding of dn-Src to caveolin-1 displaced G_{i} from caveolin-1. This could account for the observation that significantly less G_{i} coimmunoprecipitated with caveolin-1 in the cells transfected with dn-Src. The data are consistent with the model that caveolin-1 serves as a scaffolding protein for G_{i} and Src family kinases, which activate the signaling machinery mediating the gp60-induced vesicular transport. Src family tyrosine kinases activated by receptor dimerization (M. arshall, 1995) or by βγ subunits upon stimulation of G-protein-coupled receptors (Luttrell et al., 1996; Igishi and Gutkind, 1998) may phosphorylate caveolin-1 (Lisanti et al., 1994; Li et al., 1996; Tiruppathi et al., 1997) and gp60 (Tiruppathi et al., 1997), and thereby signal vesicle formation and trafficking. Thus, the gp60-caveolin-1 complex could function in much the same manner as G-protein-coupled receptors that interact with G_{i} and Src.

In summary, we have shown that G_{i} is required for signaling of vesicle formation in endothelial cells after gp60 activation. Overexpression of wt-caveolin-1 and expression of dn-Src prevented gp60-induced formation of vesicles. In each case, the role of G_{i} was important since caveolin-1-overexpressing cells sequestered G_{i} whereas dn-Src expression interfered with the binding of G_{i} to caveolin-1. These results indicate an important role of gp60-induced activation of the G_{i}-coupled Src kinase pathway in signaling the formation of endocytic vesicles and their directed migration to the basolateral surface of the vascular endothelial barrier.

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