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# Small Interfering RNA-mediated Down-regulation of Caveolin-1 Differentially Modulates Signaling Pathways in Endothelial Cells*

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**Caveolin-1 is a scaffolding/regulatory protein that interacts with diverse signaling molecules in endothelial cells. To explore the role of this protein in receptor-modulated signaling pathways, we transfected bovine aortic endothelial cells (BAEC) with small interfering RNA (siRNA) duplexes to down-regulate caveolin-1 expression. Transfection of BAEC with duplex siRNA targeted against caveolin-1 mRNA selectively "knocked-down" the expression of caveolin-1 by ~90%, as demonstrated by immunoblot analyses of BAEC lysates. We used discontinuous sucrose gradients to purify caveolin-containing lipid rafts from siRNA-treated endothelial cells. Despite the near-total down-regulation of caveolin-1 expression, the lipid raft targeting of diverse signaling proteins (including the endothelial isoform of nitric-oxide synthase, Src-family tyrosine kinases, Gαq and the insulin receptor) was unchanged. We explored the consequences of caveolin-1 knockdown on kinase pathways modulated by the agonists sphingosine-1 phosphate (S1P) and vascular endothelial growth factor (VEGF). siRNA-mediated caveolin-1 knockdown enhanced basal as well as S1P- and VEGF-induced phosphorylation of the protein kinase Akt and did not modify the basal or agonist-induced phosphorylation of extracellular signal-regulated kinases 1/2. Caveolin-1 knockdown also significantly enhanced the basal and agonist-induced activity of the small GTPase Rac. We used siRNA to down-regulate Rac expression in BAEC, and we observed that Rac knockdown significantly reduced basal, S1P-, and VEGF-induced Akt phosphorylation, suggesting a role for Rac activation in the caveolin siRNA-mediated increase in Akt phosphorylation. By using siRNA to knockdown caveolin-1 and Rac expression in cultured endothelial cells, we have found that caveolin-1 does not seem to be required for the targeting of signaling molecules to caveolae/lipid rafts and that caveolin-1 differentially modulates specific kinase pathways in endothelial cells.**

Caveolae are specialized plasmalemmal microdomains that were originally described on the surface of endothelial and epithelial cells (1, 2). First described as endocytic structures, caveolae have been identified as sites for the sequestration of diverse membrane-targeted signaling proteins (for review, see Refs. 3–5). Caveolae are characterized by the presence of the scaffolding/regulatory protein caveolin (6, 7) and by a distinctive lipid composition notable for high concentrations of cholesterol and sphingolipids; the presence of caveolin distinguishes caveolae from other "lipid raft" domains that have a similar lipid composition but do not necessarily contain caveolin.

The three caveolin isoforms in mammalian cells are 22–24-kDa integral membrane proteins; caveolin-1 and caveolin-2 are co-expressed in most cell types and are particularly abundant in endothelial cells, whereas caveolin-3 is an isoform that is specific to muscle cells (6). The most extensively characterized member of this protein family, caveolin-1, has been shown to interact with and modulate the function of many signaling proteins in caveolae, including the epidermal growth factor receptor, the endothelial isoform of nitric oxide synthase (eNOS),[1] Src family tyrosine kinases, Gα proteins, diverse serine/threonine protein kinases, and the insulin receptor (8, 9). On the other hand, caveolin-1 also interacts with acting binding cytoskeletal proteins; these interactions have been proposed to be required for the spatial organization of caveolin-associated membrane domains and caveolae internalization (10, 11).

Much of our understanding about the role of caveolin-1 in cellular signaling derives from overexpression experiments in heterologous systems. However, in cell types in which caveolin-1 is already robustly expressed, the complementary experimental approach of attenuating caveolin-1 expression has proven more challenging. Standard antisense methods have not been broadly applied (12) because of their intrinsic limitations, particularly in endothelial cells, where caveolin-1 is so abundant. Caveolin-1 knock-out mice have recently been characterized and provide a promising experimental system for exploring the role of caveolae in physiological processes (13–16). Cells derived from these mice lack caveolae membranes, and a prominent phenotype is observed in the vascular system of these mice with abnormalities in permeability and contractile functions (13, 14). However, biochemical analyses in vascular tissues prepared from these mice are hampered by the limited quantities of tissue available for analysis. In particular, the role of caveolin-1 in the regulation of receptor-modulated

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[1] The abbreviations used are: eNOS, endothelial nitric-oxide synthase; MAP, mitogen-activated protein; PI3-kinase, phosphatidylinositol 3-kinase; siRNA, small interfering RNA; Cav-1 siRNA, small interfering RNA targeted to the bovine caveolin-1 mRNA; VEGF, vascular endothelial growth factor; ERK, extracellular signal-regulated kinase; BAEC, bovine aortic endothelial cells; PBS, phosphate-buffered saline; MES, 2-(*N*-morpholino)ethanesulfonic acid; S1P, sphingosine-1 phosphate; GSK3β, glycogen synthase kinase 3β; ANOVA, analysis of variance.

kinase pathways has been difficult to ascertain, particularly with respect to the differential control of MAP kinase and phosphatidylinositol-3 (PI3) kinase pathways, both of which have regulatory components localized in caveolae (8, 17). Antisense RNA studies have implied that caveolin-1 has an inhibitory effect on the MAP-kinase cascade in NIH-3T3 cells (18). The role of caveolin-1 in the PI3-kinase/Akt pathway is more controversial; some reports suggest that overexpression of caveolin-1 down-regulates the PI3-kinase pathway and sensitizes fibroblast and epithelial cells to apoptotic stimuli (19, 20), whereas other studies have reported that overexpression of caveolin-1 activates Akt signaling (21, 22).

In this article, we describe the results of experiments using transfection of small interfering RNA (siRNA) duplexes to specifically and efficiently knockdown caveolin-1 expression in cultured bovine aortic endothelial cells (BAEC). We explore the effects of siRNA-mediated caveolin-1 knockdown on signaling pathways initiated by the agonists S1P and vascular endothelial growth factor (VEGF), and the consequence of caveolin-1 knockdown on the subcellular targeting of key signaling proteins in endothelial cells.

## EXPERIMENTAL PROCEDURES

*Materials*—Fetal bovine serum was purchased from Hyclone (Logan, UT). Cell culture reagents, media, and LipofectAMINE 2000 transfection reagent were from Invitrogen. S1P was purchased from BIOMOL (Plymouth Meeting, PA). VEGF, bradykinin, and wortmannin were from Calbiochem (La Jolla, CA). Polyclonal antibodies directed against phospho-eNOS (Ser-1179), phospho-Akt (Ser-473), Akt, phospho-glycogen synthase kinase 3$\beta$ (GSK3$\beta$) (Ser-9), phospho-ERK1/2 (Thr-202/Tyr-204) and ERK1/2 were from Cell Signaling Technologies (Beverly, MA). Caveolin-1 polyclonal antibody, eNOS monoclonal antibody, GSK3$\beta$ monoclonal antibody, and flotillin-1 monoclonal antibody were from BD Transduction Laboratories. Polyclonal antibodies for Src, the insulin receptor ($\beta$ subunit), and G$\alpha$q were from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488 anti-mouse IgG antibody, Alexa Fluor 568 anti-rabbit IgG antibody, anti-Alexa Fluor 488 antibody, bovine serum albumin labeled with Alexa Fluor 488, phalloidin labeled with Alexa Fluor 488 and 4′,6-diamino-2-phenylindole, dihydrochloride were from Molecular Probes Inc. (Eugene, OR). Rac activation assay kit was from Upstate Biotechnology (Lake Placid, NY). Super Signal substrate for chemiluminescence detection and secondary antibodies conjugated with horseradish peroxidase were from Pierce. Tris-buffered saline and phosphate-buffered saline were from Boston Bioproducts (Ashland, MA). Other reagents were from Sigma.

*Cell Culture*—Bovine aortic endothelial cells (BAEC) were obtained from Cell Systems (Kirkland, WA) and maintained in culture in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10% v/v) as described previously (23). Cells were plated onto 0.2% gelatin-coated culture dishes and studied before cell confluence between passages 5 and 9.

*siRNA Preparation and Transfection*—Based upon established characteristics of siRNA targeting constructs (24, 25), we designed a caveolin-1 siRNA duplex corresponding to bases 223–241 from the open reading frame of the bovine caveolin-1 mRNA: 5′-CCA GAA GGA ACA CAC AGU U-dTdT-3′, and a Rac siRNA duplex corresponding to bases 78–96 from the open reading frame of the bovine Rac mRNA: 5′-UGC GUU UCC UGG AGA AUA U-dTdT-3′. The RNA sequence used as a negative control for siRNA activity was: 5′-GCG CGC UUU GUA GGA UUC G-dTdT-3′. Small interfering RNA duplex oligonucleotides were purchased from Dharmacon, Inc. (Lafayette, CO). In preliminary experiments, we optimized conditions for the efficient transfection of BAEC using siRNA. We found that optimal conditions for siRNA knockdown involved transfecting BAEC at 50–70% confluence maintained in Dulbecco's modified Eagle's medium/10% fetal bovine serum; transfections with siRNA (30 nM, or as noted) used LipofectAMINE 2000 (0.15%, v/v), following protocols provided by the manufacturer. Fresh medium was added 5 h after transfection, and experiments were conducted 48 h after transfection.

*Drug Treatment, Cell Lysates, and Immunodetection*—Culture medium was changed to serum-free medium, and incubations proceeded overnight before all experiments (26, 27).

S1P was solubilized in methanol and stored at −20 °C; the same volume of methanol was used as a vehicle control, and the final concentration of methanol did not exceed 0.4% (v/v). VEGF was solubilized in Tris-buffered saline containing 0.1% bovine serum albumin and stored at −70 °C. Bradykinin was solubilized in water and stored at −20 °C.

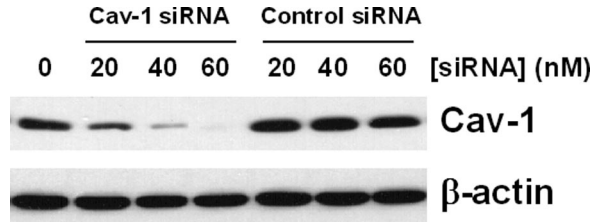


FIG. 1. **SiRNA-mediated down-regulation of caveolin-1 expression in BAEC.** BAEC were transfected with the indicated concentrations of duplex siRNA targeted against caveolin-1 (*Cav-1 siRNA*) or a random sequence (*Control siRNA*). 48 h after transfection, cells were harvested and lysed, and protein levels were analyzed in immunoblots probed with a caveolin-1 antibody or $\beta$-actin antibody, as shown.

After drug treatments, BAEC were washed with phosphate-buffered saline (PBS) and incubated for 10 min in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 2 mM $Na_3VO_4$, 1 mM NaF, 2 $\mu$g/ml leupeptin, 2 $\mu$g/ml antipain, 2 $\mu$g/ml soybean trypsin inhibitor, and 2 $\mu$g/ml lima trypsin inhibitor). Cells were harvested by scraping and then centrifuged for 15 min at 4 °C. For immunoblot analyses, 20 $\mu$g of cellular protein were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed with antibodies using protocols provided by the suppliers. Densitometric analyses of the Western blots were performed using a ChemiImager 4000 (Alpha Innotech).

*Rac Activity Assay*—BAEC in 100-mm dishes were transfected with control or caveolin-1-specific siRNA. Cells were starved overnight before experiments, and Rac activity was measured 48 h after transfection. After stimulation with S1P, cells were washed with ice-cold PBS and lysed in lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 2 mM $Na_3VO_4$, 1 mM NaF, 2 $\mu$g/ml leupeptin, 2 $\mu$g/ml antipain, 2 $\mu$g/ml soybean trypsin inhibitor, and 2 $\mu$g/ml lima trypsin inhibitor). Pull-down of GTP-bound Rac was performed by incubating the cell lysates with glutathione *S*-transferase fusion-protein corresponding to the p21-binding domain of PAK-1 bound to glutathione agarose (Upstate Biotechnology) for 1 h at 4 °C following instructions provided by the suppliers. The beads were washed three times with lysis buffer, and the protein bound to the beads was eluted with Laemmli buffer and analyzed for the amount of GTP-bound Rac by immunoblotting using a Rac monoclonal antibody (Upstate Biotechnology).

*Migration Assay*—Cell migration was assayed (30) using a Transwell cell culture chamber containing polycarbonate membrane inserts with 8-$\mu$m pore (Corning Costar Corp.) coated with 0.2% gelatin. BAEC were transfected with control or caveolin-1-specific siRNA, and experiments were carried out 48 h after transfection; the transfected cells were maintained in Dulbecco's modified Eagle's medium supplemented with 0.4% fetal bovine serum for 18 h before migration experiments. The cells were briefly incubated with trypsin to obtain a single-cell suspension, and $5 \times 10^4$ cells in Dulbecco's modified Eagle's medium/0.4% fetal bovine serum were added to the upper Transwell chamber. The bottom chamber was filled with 600 $\mu$l of media, and the assembly was incubated at 37 °C for 1 h. S1P (100 nM) was added to the lower chamber, and the assembly was incubated at 37 °C for 3 h to allow cell migration. In some experiments, wortmannin (500 nM) was added to the lower chamber 30 min before the addition of S1P. After incubation, the membranes were washed with PBS, and the cells were fixed in 3.7% formaldehyde (10 min), permeabilized in 0.5% Triton X-100 in PBS (3 min), and stained with 4′,6-diamino-2-phenylindole, dihydrochloride according to the manufacturer's protocols. Cells that did not migrate through the membrane were gently removed from the upper surface and the membranes were mounted between slide and coverslips using the SlowFade antifade kit (Molecular Probes Inc.) Cell migration was scored under light microscopy in four random fields by a blinded observer; each experimental treatment was analyzed in triplicate.

*Albumin Uptake*—BAEC were transfected with control or caveolin-1-specific siRNA; 48 h after transfection, cells were incubated with Alexa Fluor 488-conjugated albumin (10 $\mu$g/ml) at 37 °C. Cells were washed with PBS, and cell lysates were prepared as described above. Albumin uptake was analyzed in immunoblots probed with an antibody against Alexa Fluor 488.

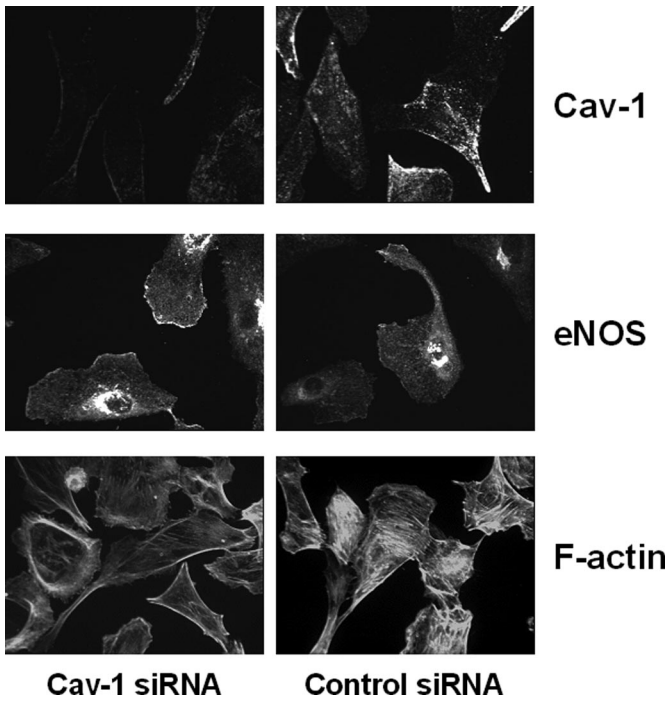


FIG. 2. **Confocal microscopy analyses of caveolin-1, eNOS, and F-actin distribution in caveolin-1 siRNA-treated BAEC.** BAEC were transfected with 30 nM siRNA targeted against caveolin-1 or control siRNA. 48 h after transfection, cells were fixed and stained for caveolin-1 or eNOS immunofluorescence using specific antibodies or for F-actin using Alexa Fluor 488-labeled phalloidin, as noted. Images are single optical slices in the *z*-axis.

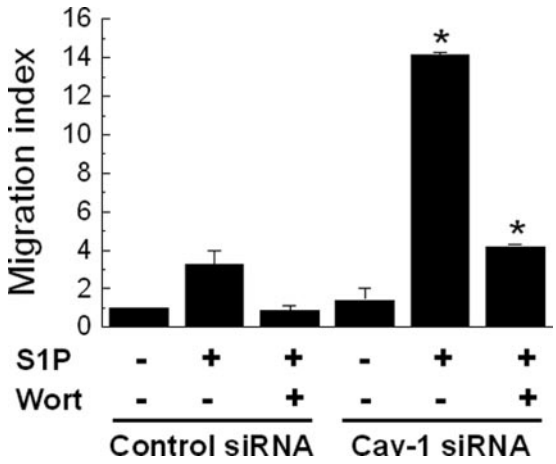


FIG. 3. **Effects of siRNA-mediated caveolin-1 knockdown on endothelial cell migration.** Endothelial cell migration was measured using a Transwell system in BAEC transfected with control or caveolin-1 siRNA and treated with S1P (100 nM) in the presence or absence of wortmannin (*Wort*; 500 nM). *Migration index* represents the number of migratory cells/number of migratory cells in vehicle-treated control siRNA-transfected BAEC. Each data point represents the mean ± S.E. of triplicate determinations. *, $p < 0.01$ *versus* control siRNA transfected cells (using ANOVA).

*Isolation of Caveolae-enriched Fractions*—Caveolae-enriched fractions were separated by ultracentrifugation in a discontinuous sucrose gradient system as reported previously (28). In brief, transfected BAEC from two 100-mm dishes were scraped together into 2 ml of carbonate buffer containing 500 mM sodium carbonate, pH 11, and the cells were homogenized (40 strokes in a Dounce homogenizer) and sonicated (three 20-s bursts in a Branson Sonifier 450). The resulting cell suspension was brought to 45% sucrose by adding 2 ml of 90% sucrose prepared in MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl) and placed at the bottom of a 12-ml ultracentrifuge tube. A discontinuous gradient was formed above the 45% sucrose bed by adding 4 ml

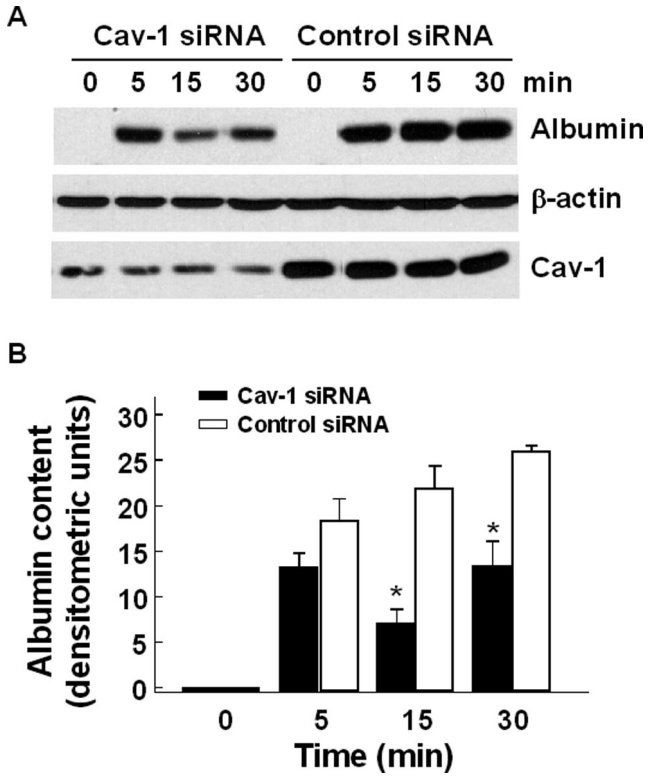


FIG. 4. **Albumin uptake in caveolin-1 siRNA-treated BAEC.** *A*, BAEC transfected with control or caveolin-1 siRNA were incubated with Alexa Fluor 488-conjugated albumin (10 $\mu$g/ml) for the indicated times. After washing, cells were harvested and total cell lysates were analyzed in immunoblots using an antibody against Alexa Fluor 488. $\beta$-Actin protein levels are shown as a loading control. *B*, results of pooled data (mean ± S.E.) from three such independent experiments analyzed by densitometry. *, $p < 0.01$ *versus* control cells (using ANOVA).

each of 35% and 5% sucrose solutions (prepared in MES-buffered saline containing 250 mM of sodium carbonate) and centrifuging at 39,000 rpm for 16–20 h in a TH-641 rotor (Sorvall, Asheville, NC). Twelve 1-ml fractions were collected starting at the top of each gradient, and an equal volume of each fraction was analyzed by SDS-PAGE and immunoblotting as described above.

*Confocal Fluorescence Microscopy*—BAEC grown on coverslips were transfected with control or caveolin-1-specific siRNA using LipofectAMINE 2000. 48 h after transfection, cells were fixed with 3.7% paraformaldehyde in PBS for 10 min, rinsed with PBS, permeabilized in 0.1% Triton X-100/0.1% bovine serum albumin in PBS for 5 min, and blocked with 10% horse serum in PBS for 1 h. Incubations with primary antibodies were performed in blocking solution for 1 h at room temperature. After washing with PBS, cells were incubated with appropriate secondary antibodies conjugated to immunofluorescent dyes (Alexa Fluor 488 anti-mouse IgG or Alexa Fluor 568 anti-rabbit IgG) in blocking solution for 1 h at room temperature. After washing three times with PBS, coverslips were mounted on slides using the SlowFade antifade kit (Molecular Probes Inc.). For staining of F-actin, fixed cells were permeabilized in 0.1% Triton X-100 in PBS for 3 min and blocked with 1% bovine serum albumin in PBS for 30 min. Cells were incubated with phalloidin-Alexa Fluor 488 in PBS containing 1% bovine serum albumin for 20 min at room temperature. After washing with PBS, the coverslips were mounted on slides as described previously. Microscopy analysis of samples was performed in the Nikon Imaging Center at Harvard Medical School, using a Nikon TE2000U inverted microscope in conjunction with a PerkinElmer Ultraview spinning disk confocal system equipped with a Hamamatsu Orca ER cooled-CCD camera. Images were acquired using a 60× differential interference contrast oil immersion objective lens and analyzed using Metamorph software from Universal Imaging, Inc. (Downingtown, PA).

## RESULTS

*siRNA-mediated Down-regulation of Caveolin-1 Expression in BAEC*—To selectively "knockdown" the expression of caveo-

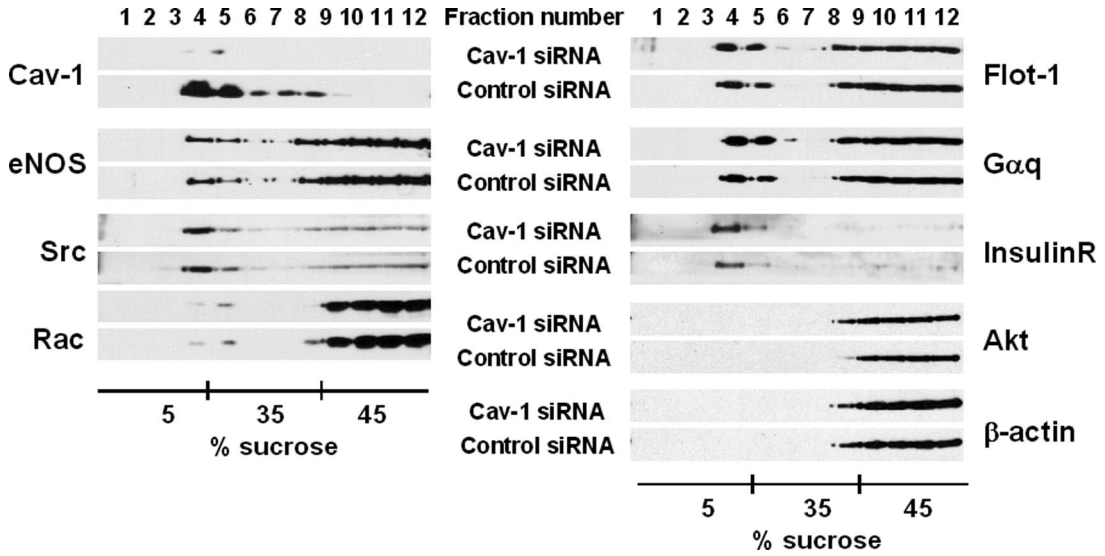


FIG. 5. **Subcellular fractionation of signaling proteins after siRNA-mediated caveolin-1 knockdown.** BAEC transfected with caveolin-1 or control siRNA were subjected to subcellular fractionation using ultracentrifugation in a discontinuous sucrose gradient. An aliquot of each fraction was resolved by SDS-PAGE and analyzed in immunoblots probed with specific antibodies as shown. The caveolae/lipid raft fraction of this sucrose gradient corresponds to *fractions 4* and *5*, whereas cytosolic proteins and the bulk of cellular membrane proteins remain in *fractions 9–12*. Shown are the results of a representative experiment that was repeated three times with equivalent results.

lin-1 protein in BAEC, we designed a siRNA duplex targeted to the bovine caveolin-1 mRNA (Cav-1 siRNA, sequence 5′-CCAGAAGGAACACACAGUU-dTdT-3′, corresponding to bases 223–241 from the open reading frame of bovine caveolin-1 mRNA). We decided to use siRNA duplexes rather than plasmid-based RNA interference techniques because preliminary experiments (and our past experience) showed that plasmid-based methods had an unacceptably low transfection efficiency in endothelial cells. After optimizing conditions for duplex siRNA transfection, we assayed the ability of this caveolin-1-specific siRNA to knock down caveolin expression by transfecting BAEC with increasing concentrations of this siRNA duplex. Fig. 1*A* shows an immunoblot probed for caveolin-1 protein in BAEC 48 h after transfection with caveolin-1-specific siRNA. Caveolin-1 expression was efficiently knocked-down in a dose-dependent manner by transfection with Cav-1 siRNA; transfection with a control random sequence siRNA did not affect the expression of caveolin-1 (Fig. 1). Under both experimental conditions, levels of β-actin protein in cell lysates remained constant.

To further characterize the effect of caveolin-1 siRNA on caveolin-1 expression levels and protein distribution, we performed immunofluorescence analysis of caveolin-1 in BAEC transfected with Cav-1 and control siRNA. As illustrated in Fig. 2, cells transfected with caveolin-1 siRNA showed a marked reduction of caveolin-1 expression levels compared with control cells (Fig. 2). The subcellular distribution pattern of the small amount of residual caveolin-1 remaining after siRNA-mediated knockdown paralleled the caveolin-1 distribution pattern of control cells: despite the quantitative reduction in caveolin-1 expression, the distribution of the expressed protein seemed to be unchanged (Fig. 2). We next explored whether down-regulation of caveolin-1 expression would affect the subcellular distribution of caveolae-targeted proteins. Fig. 2 shows the results of immunofluorescence microscopy analyses of BAEC transfected with control or caveolin-1 siRNA and stained for the key caveolae-targeted signaling protein eNOS. The subcellular distribution of eNOS was not affected by siRNA-mediated knockdown of caveolin-1; the enzyme was detected as a membrane-associated protein localized at the plasma membrane and around the perinuclear region, as described previously in native BAEC (29). Because caveolin-1 has been reported to interact with a variety of actin-binding cytoskeletal proteins (10, 11), we also explored whether actin organization would be affected in caveolin-1 knockdown cells. Fig. 2 shows siRNA-transfected BAEC stained with Alexa Fluor 488-labeled phalloidin to visualize the actin cytoskeleton. As seen in this figure, caveolin-1 siRNA-treated cells showed both a notable increase in the abundance of lamellipodia and augmented cortical actin rings compared with control cells, suggesting that cytoskeletal structure is altered in endothelial cells in which caveolin-1 is knocked down.

*Down-regulation of Caveolin-1 Expression Enhances S1P-induced Migration of BAEC*—Because siRNA-mediated down-regulation of caveolin-1 significantly altered the cytoskeletal structure of BAEC, we next explored whether caveolin-1 knockdown affected agonist-induced endothelial cell migration. Fig. 3 shows the results of a cell motility assay using a Transwell cell culture chamber. As reported previously in wild-type BAEC (30), we observed a chemotactic response to S1P in BAEC treated with the control siRNA. BAEC transfected with caveolin-1-specific siRNA displayed significantly greater S1P-mediated migration compared with control cells. The PI3-kinase inhibitor wortmannin significantly attenuated S1P-induced cell migration in control and caveolin-1 siRNA-treated cells (Fig. 3), suggesting that the augmentation in BAEC migration induced by caveolin-1 siRNA is mediated by PI3-kinase pathways.

*Down-regulation of Caveolin-1 Expression Impairs Albumin Uptake in BAEC*—Previous reports have suggested that the transport of albumin across the endothelial barrier is mediated by caveolae (31, 14). We analyzed the effects of siRNA-mediated caveolin-1 knockdown on albumin uptake in cultured BAEC. Control or caveolin-1 siRNA transfected BAEC were incubated with Alexa Fluor 488-labeled albumin, and albumin uptake was analyzed in the cell lysates at different time points by immunoblot analyses using an antibody against the exogenous labeled albumin. As shown in Fig. 4, the uptake of exogenous albumin was significantly attenuated in caveolin-1 siRNA-treated cells, demonstrating that down-regulation of caveolin-1 expression is sufficient to impair caveolae-mediated albumin endocytosis in BAEC.

*Down-regulation of Caveolin-1 Expression Does Not Impair Targeting of Signaling Molecules*—Diverse signaling molecules

are targeted to caveolae (5), where caveolin-1 represents the dominant protein component and is essential for the structure of these plasma membrane microdomains (6, 7, 14). Lipid rafts have a lipid composition similar to caveolae, but caveolae are distinguished from non-caveolar lipid rafts by the presence of caveolin (7, 32). Both caveolae and lipid rafts have a distinctive lipid composition, in that they are enriched in cholesterol and sphingolipids and therefore characterized by a low buoyant density, which facilitates their subcellular fractionation. As shown by immunofluorescence staining, the subcellular distribution of the caveolae-targeted signaling molecule eNOS was not affected by siRNA-mediated caveolin-1 knockdown (Fig. 2). We also used a hydrodynamic approach to examine the effects of caveolin-1 down-regulation on the targeting of a broad range of signaling molecules to caveolin-enriched/lipid raft microdomains. Cell lysates from BAEC transfected with control or caveolin-1 siRNA were analyzed by ultracentrifugation using a discontinuous sucrose gradient system that was previously shown to resolve lower-density lipid rafts and caveolae from cytosolic and plasma membrane proteins (28, 33). As shown in Fig. 5, caveolin-1 was mostly recovered from control cells at the interface between the 5 and 35% sucrose solutions (fractions 4 and 5). Although there was a marked reduction in the quantity of caveolin-1 in fractions prepared from caveolin-1 siRNA-treated cells, the remaining caveolin-1 showed a distribution in sucrose gradient fractionation similar to that of caveolin-1 in control siRNA-treated cells. As shown also in Fig. 5, caveolin-1 knockdown did not alter either the abundance or the subcellular fractionation pattern of diverse signaling proteins, including caveolae-targeted signaling proteins (such as flotillin-1 (34), eNOS (35, 36), G$\alpha$q (37), c-Src (38), Rac (39, 40), and the insulin receptor (41)) as well as soluble cellular proteins and proteins associated with dense membrane fractions. Despite a significant decrease in caveolin-1 expression after caveolin-1 siRNA transfection of BAEC, neither the expression level nor the subcellular distribution of these diverse proteins was affected.

*Caveolin-1 Knockdown Potentiates Akt Phosphorylation without Modifying ERK1/2 Phosphorylation*—Caveolin-1 has been found previously to modulate diverse signaling cascades by interacting with and inhibiting a broad range of signaling proteins (for review, see Ref. 8). These protein interactions have been characterized principally by examining the consequences of caveolin-1 overexpression in heterologous cell systems. Here, we exploited siRNA-mediated down-regulation of caveolin-1 expression to explore the role of caveolin-1 in agonist-induced activation of the PI3-kinase/Akt and MAP kinase pathways. BAEC were transfected with control or caveolin-1 siRNA; 48 h after transfection, the cells were stimulated with one of the vascular agonists S1P, VEGF, or bradykinin. Cell lysates were analyzed in immunoblots probed with phospho-specific antibodies generated against activated (phosphorylated) protein kinases Akt and ERK1/2, which are downstream effectors of these receptor pathways. As shown in Fig. 6*A*, cells treated with caveolin-1 siRNA showed a marked increase in Akt phosphorylation relative to control transfected cells. A significant increase in Akt phosphorylation was observed both in the basal state (2.4 ± 0.4-fold, $p < 0.01$) and after treatment with S1P (2.0 ± 0.2-fold, $p < 0.01$) or VEGF (1.9 ± 0.2-fold, $p < 0.01$). Bradykinin stimulation did not alter the level of Akt phosphorylation in either control or caveolin-1 down-regulated BAEC. There was no change in the expression of total Akt, ERK1/2, or $\beta$-actin under any of these conditions (Fig. 6*A*), which further validated the specificity of siRNA-mediated caveolin-1 knockdown. Treatment with the PI3-kinase inhibitor wortmannin before adding S1P or VEGF completely blocked Akt phosphorylation in both control and caveolin-1 siRNA-treated BAEC (data not shown), indicating that enhanced Akt phosphorylation in caveolin-1 knockdown cells required PI3-kinase activation. Moreover, there was no effect of caveolin-1 knockdown on the abundance or phosphorylation of the phospholipid phosphatase PTEN or of the phosphoinositide-dependent kinase PDK1 (data not shown). In contrast to the enhanced Akt phosphorylation induced by caveolin-1 siRNA, there were no significant differences in either basal or agonist-induced ERK1/2 phosphorylation levels between control and caveolin-1 siRNA-treated BAEC (Fig. 6*B*). This finding suggests that caveolin-1 knockdown does not affect basal or agonist-depend-

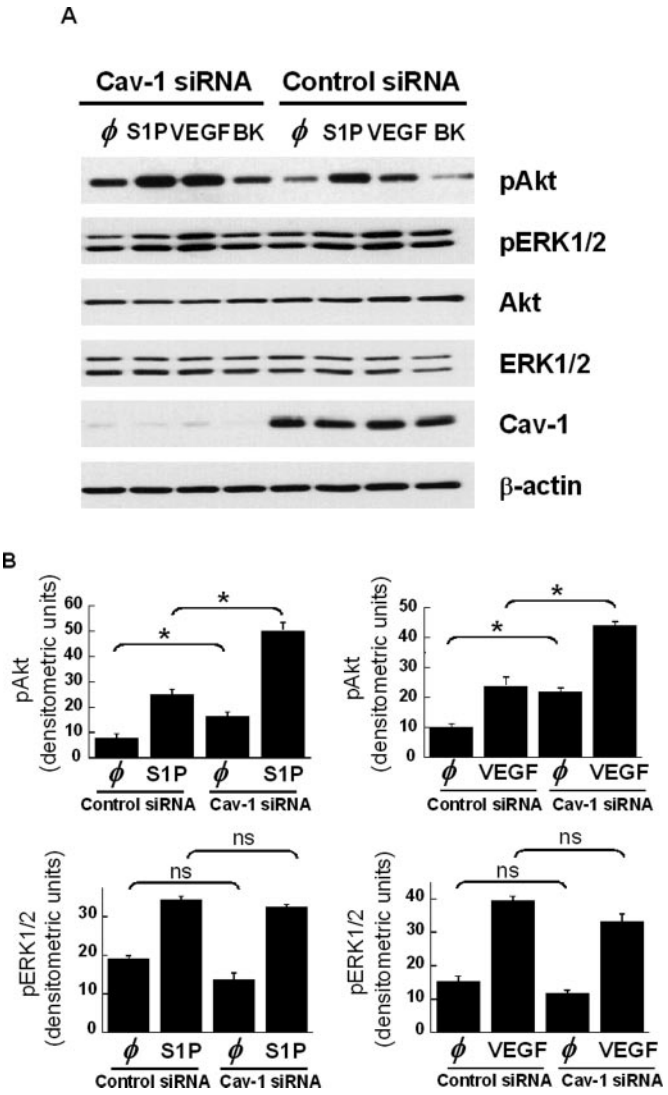


FIG. 6. **Effects of siRNA-mediated caveolin-1 knockdown on PI3-kinase and MAP-kinase pathways in BAEC.** *A*, an immunoblot prepared from BAEC transfected with siRNA targeted against caveolin-1 or control siRNA and treated with S1P (100 nM), VEGF (20 ng/ml), or bradykinin (*BK*; 1 $\mu$M) for 5 min. The cell lysates were resolved by SDS-PAGE and analyzed in immunoblots probed with antibodies against phospho-Akt, phospho-ERK1/2, Akt and ERK1/2. Caveolin-1 and $\beta$-actin expression were quantified in immunoblots probed with their respective antibodies. *B*, the results of densitometric analyses from pooled data, showing the level of Akt and ERK1/2 phosphorylation in caveolin-1 siRNA or control siRNA-transfected cells in basal conditions and after treatment with S1P or VEGF. Each data point represents the mean ± S.E. derived from five independent experiments; under all conditions, the addition of either S1P or VEGF induced a significant increase in phosphorylation of Akt and ERK1/2 ($p < 0.01$). *, $p < 0.01$ for caveolin-1 *versus* control siRNA transfected cells (determined by ANOVA). *ns*, not significant; *ϕ*, vehicle.

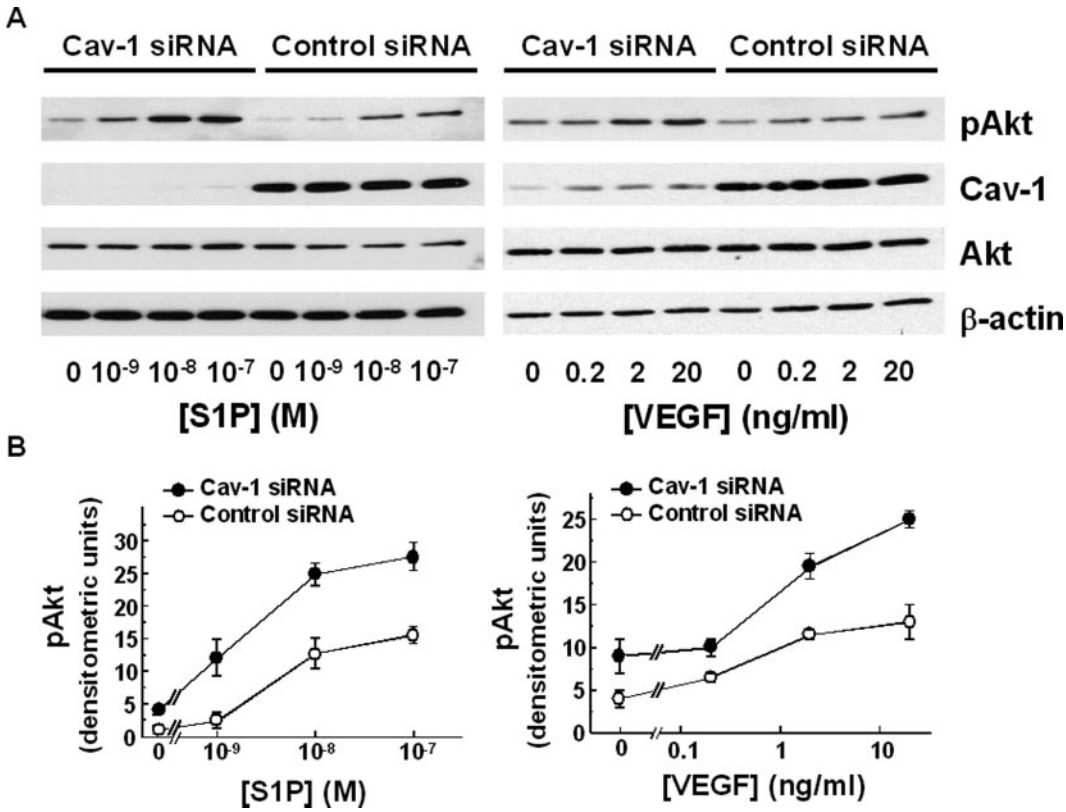


FIG. 7. **Dose response of S1P- and VEGF-induced Akt phosphorylation in caveolin-1 siRNA-treated BAEC.** *A*, BAEC transfected with caveolin-1-specific siRNA or control siRNA were treated with the indicated concentrations of S1P (*left*) or VEGF (*right*) for 5 min. After the treatments, aliquots of cell lysates (20 $\mu$g/lane) were resolved by SDS-PAGE and analyzed in immunoblots probed with phospho-Akt antibody. Equal loading was confirmed by immunoblotting with anti-Akt and anti-$\beta$-actin antibodies. Caveolin-1 knockdown was assayed by immunoblotting with anti-caveolin-1 polyclonal antibody. Shown are the representative results of an experiment that was repeated three times with equivalent results. *B*, pooled data in which the signal intensity of each data point was analyzed by densitometry. Each *point* in the graph represents the mean ± S.E. of three independent experiments.

ent activation of the Ras/p44/42 MAP-kinase pathway in endothelial cells.

To better characterize the effects of caveolin-1 in the activation of the protein kinase Akt, we analyzed the dose-response and time-course for S1P- and VEGF-induced Akt phosphorylation. As shown in Fig. 7, these agonists promoted dose-dependent increases in Akt phosphorylation, with $EC_{50}$ values of ~2 nM for S1P and ~1 ng/ml for VEGF in control cells. siRNA-mediated caveolin-1 knockdown potentiated the agonist-induced phosphorylation of Akt at every dose analyzed without significantly affecting the $EC_{50}$ values. Loading controls (shown below in each figure) documented equivalent levels of Akt protein expression under these different experimental conditions. As shown in Fig. 8, similar time courses of agonist-induced Akt activation were observed in control and caveolin-1 siRNA-treated BAEC, although Akt phosphorylation was enhanced in caveolin-1 siRNA-treated cells at each time point analyzed.

*Up-regulation of Akt Phosphorylation in Caveolin-1 Knockdown BAEC Differentially Affects Downstream Targets of Akt*—We next explored whether enhanced Akt phosphorylation in caveolin-1 down-regulated cells would affect the phosphorylation of two known Akt substrates, eNOS and GSK3$\beta$. BAEC were transfected with control or caveolin-1-specific siRNA and stimulated with S1P or VEGF 48 h after transfection. Phosphorylation of the Akt substrates eNOS (phosphorylated at Ser-1179; Refs. 42, 43) and GSK3$\beta$ (phosphorylated at Ser-9; Ref. 44) was analyzed in immunoblots probed with the respective phosphospecific antibodies. As shown in Fig. 9, no significant differences were observed in eNOS phosphorylation at serine 1179 under these experimental conditions, despite an increase in basal and agonist-induced phosphorylation of Akt in caveolin-1 siRNA-treated cells. By contrast, caveolin-1 siRNA-treated cells showed markedly enhanced GSK3$\beta$ phosphorylation, both in the basal state (1.9 ± 0.1-fold increase relative to control cells, $p < 0.001$) and after treatment with S1P (1.5 ± 0.05-fold increase, $p < 0.001$) or VEGF (1.4 ± 0.05-fold increase, $p < 0.001$). These findings suggest that the enhancement of Akt phosphorylation by caveolin-1 siRNA differentially affects the phosphorylation pattern of discrete Akt downstream effectors.

*Caveolin-1 Knockdown Enhances Rac Activity in Endothelial Cells*—Members of the small GTPase Rho family, including Rho, Rac, and cdc42, regulate the reorganization of the actin cytoskeleton and are considered critical determinants of endothelial barrier function (45, 46). We were intrigued to note that after down-regulation of caveolin-1 expression in BAEC, there seemed to be an increase in lamellipodial structures as well as enhanced cortical actin distribution (Fig. 2); these cellular features are known to be stimulated by activated Rac (46). Several studies have previously shown that Rac is concentrated in caveolae in different cell types, including fibroblasts and cardiac myocytes (39, 40); it is noteworthy that we found that a small fraction of Rac is also targeted to caveolae/lipid raft domains in endothelial cells (Fig. 5). We therefore explored whether caveolin-1 knockdown might induce Rac activation. BAEC were transfected with control or caveolin-1-specific siRNA and stimulated with S1P 48 h after transfection. GTP-bound Rac was isolated from endothelial cell lysates using the glutathione *S*-transferase-fused p21-binding domain of PAK-1. GTP-bound Rac and total Rac in the cell lysates were quantified in immunoblots probed with a monoclonal antibody di-

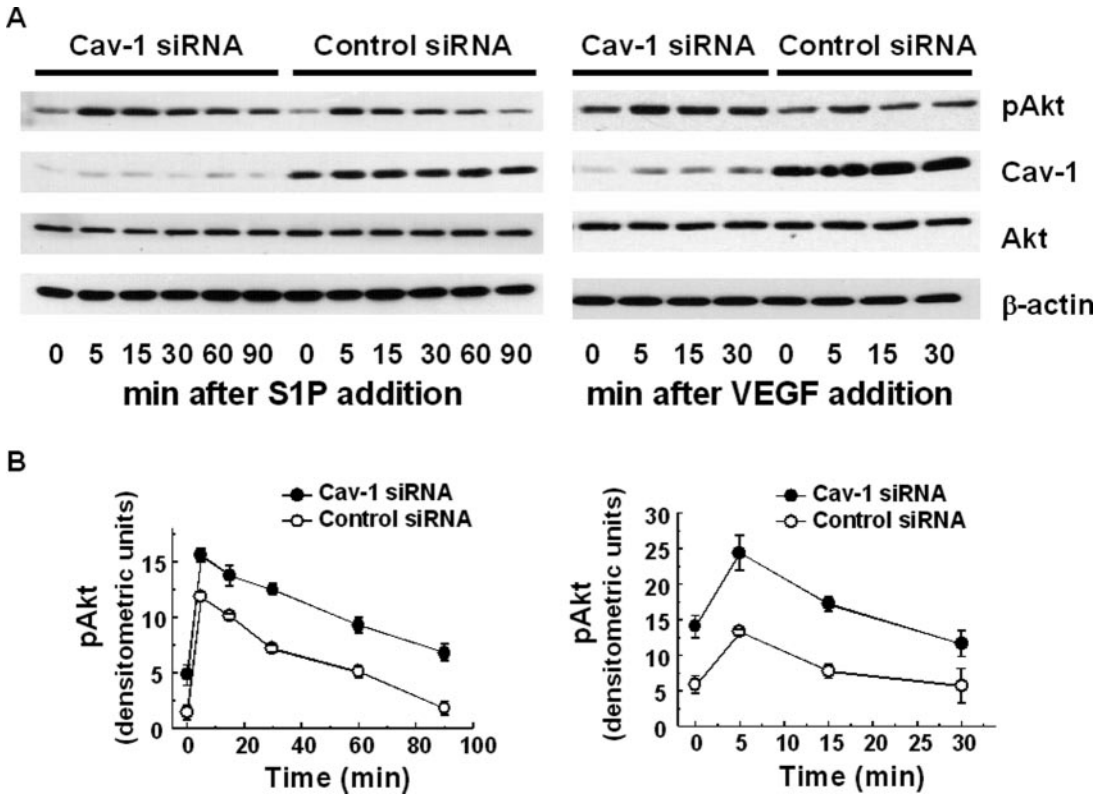


FIG. 8. **Time course of S1P- and VEGF-induced Akt phosphorylation in caveolin-1 siRNA-treated BAEC.** *A*, BAEC were transfected with caveolin-1 siRNA or control siRNA; 48 h after transfection, cells were treated with S1P (100 nM) or VEGF (20 ng/ml) for the indicated times. Cell lysates were resolved by SDS-PAGE and analyzed in immunoblots probed with phospho-Akt specific antibody. Equal loading of the samples was confirmed by immunoblot with Akt- and β-actin-specific antibodies, as shown. Caveolin-1 expression was analyzed in immunoblots with anti-caveolin-1 antibody. Shown are the representative results of an experiment that was repeated three times with equivalent results. *B*, pooled data, plotting the signal intensity of each data point as determined by densitometry. Each point in the graph represents the mean ± S.E. of three independent experiments.

rected against Rac. As shown in Fig. 10, cells treated with caveolin-1-specific siRNA showed a significant increase in GTP-bound Rac compared with control siRNA-transfected cells, both in the basal state (4.2 ± 1.5-fold increase, $p < 0.05$) and after S1P stimulation (1.9 ± 0.2-fold increase, $p < 0.001$), suggesting that caveolin-1 acts as a negative modulator of Rac in BAEC.

*Rac Knockdown Inhibits Akt Phosphorylation in BAEC*—Previous studies have shown that active mutants of the small GTPase Rac enhanced PI3-kinase activity and induced Akt phosphorylation in COS-7 and NIH3T3 cells (47, 48). We explored whether Rac could act as an activator of the PI3-kinase/Akt pathway in endothelial cells and thereby provide a mechanism for the enhancement of Akt phopshorylation observed in caveolin-1 siRNA-treated cells. We designed an siRNA targeted against bovine Rac mRNA (Rac siRNA), and we analyzed the silencing capacity of this siRNA by transfecting BAEC with increasing concentrations of Rac siRNA duplex. As shown in Fig. 11, Rac expression was efficiently knocked down in a dose-dependent manner 48 h after transfection with Rac siRNA. Transfection with a control siRNA did not affect the expression of Rac, and levels of β-actin remained constant in the cell lysates under both experimental conditions. We next explored the effect of Rac knockdown in agonist-induced activation of the PI3- and MAP-kinase pathways. BAEC were transfected with Rac or control siRNA; 48 h after transfection, S1P- or VEGF-induced phosphorylation of protein kinase Akt and ERK1/2 was analyzed in immunoblots probed with phosphospecific antibodies. As shown in Fig. 12, siRNA-mediated down-regulation of Rac expression significantly attenuated Akt phosphorylation both in the basal state (12.5 ± 2.9-fold, $p < 0.01$) and after treatment with S1P (2.4 ± 0.5-fold, $p < 0.01$) or VEGF (2.8 ± 0.5-fold, $p < 0.01$). By contrast, siRNA-mediated Rac knockdown affected neither basal nor agonist-induced ERK1/2 phosphorylation levels in BAEC. These results provide evidence of Rac acting as an upstream modulator of protein kinase Akt in endothelial cells.

## DISCUSSION

These studies have successfully adapted siRNA-based methods to study the roles of the scaffolding/regulatory protein caveolin-1 in vascular endothelial cell signaling pathways. Caveolin-1 is robustly expressed in endothelial cells, where the protein has been postulated to modulate cellular responses (49). However, much of what is known or conjectured about caveolin-1 in endothelial signal transduction is based upon overexpression studies in heterologous cell systems. These approaches, although informative (and extensively pursued by our lab and by many others), are limited by the challenges inherent in establishing the biological relevance of observations made in heterologous overexpression systems. Previous studies of caveolin-1 down-regulation in endothelial cells using standard antisense RNA methods reduced the endogenous protein expression by only ~50% (12), and have not been more broadly informative. The development of caveolin-1 knockout mice (13–15) provided a useful tool to study the functions of caveolin-1 *in vivo*, but biochemical studies in endothelial tissue derived from these mice are difficult because of the limited amount of protein that can be obtained for analyses. Duplex siRNA-based methods have the advantage of being able to nearly abrogate caveolin-1 expression and permit the molecular consequences to be explored in a well characterized, cul-

tured, native endothelial cell system. Moreover, the short-term and transient effects of siRNA-mediated caveolin-1 down-regulation provide an alternative experimental approach to study the functions of caveolin-1, avoiding the possible effect of compensatory mechanisms developed by caveolin-1 knockout mice. Therefore, studies performed in these two model systems may provide complementary information about the effects of short- and long-term down-regulation of caveolin-1 in endothelial cell signaling.

The present studies establish that transfection of caveolin-1 siRNA duplexes dramatically attenuates caveolin-1 levels (Figs. 1 and 2) without affecting the expression of a wide variety of endothelial signaling proteins (Fig. 5), demonstrating the specificity of this gene silencing approach. Very recently, other groups have also successfully applied siRNA methods to down-regulate caveolin-1 expression levels in astrocytes (50) and in human umbilical vein endothelial cells (51) and have studied the effects of caveolin-1 knockdown on chemotactic and endocytic responses. We also found that siRNA-mediated knockdown of caveolin-1 expression impairs albumin uptake in endothelial cells (Fig. 4), indicating that loss of caveolin-1 is sufficient to attenuate albumin endocytosis in the endothelium, as suggested by prior studies using chemical (52) or genetic (31) methods. Recent mechanistic studies further support a role for caveolin-1 in endothelial transcytosis and report that the uptake of albumin is mediated by tyrosine-kinase dependent pathways involving caveolin-1 phosphorylation (53, 54).

A major function assigned to caveolae is the compartmentalization and regulation of signaling cascades (8, 55). Biochemical studies have shown that a variety of signaling proteins concentrated within these plasma membrane microdomains interact with caveolin-1 (8). We were intrigued to find in the present studies that siRNA-mediated caveolin-1 knockdown did not seem to alter the targeting of several other caveolae-localized signaling proteins, including eNOS, Rac, G$\alpha$q proteins, tyrosine kinase Src, and the insulin receptor (Fig. 5). Confocal microscopy analyses of eNOS immunofluorescence in control and caveolin-1 siRNA treated-BAEC showed a similar subcellular distribution pattern (Fig. 2). These data complement and extend earlier observations made in a heterologous overexpression system, in which caveolin overexpression did not alter eNOS targeting (56). Electron microscopy analysis of cells derived from caveolin-1-null mice demonstrates that caveolin-1 expression is required for caveolae to form (13–15). It is therefore plausible that the formation of anatomically defined caveolae is impaired after siRNA-mediated caveolin-1 knockdown, but our data clearly indicate that the subcellular targeting of a broad range of endothelial caveolae-targeted signaling proteins is *not* dependent upon the presence of caveolin-1 (Fig. 5). We speculate that the interaction of these signaling molecules with caveolin-1 does not serve as a fundamental mechanism for protein trafficking and localization.

Numerous prior studies have suggested the *regulatory* importance of the dynamic interactions between caveolin-1 and signaling proteins (8, 33, 57, 58), and we also explored the effects of caveolin-1 knockdown on receptor-modulated kinase pathways in endothelial cells. We studied the effects of caveolin-1 siRNA on responses to the well characterized agonists S1P, VEGF, and bradykinin, which agonists have been previously found to modulate diverse kinase pathways in endothelial cells. siRNA-mediated caveolin-1 knockdown did not significantly affect basal or agonist-induced activation of the Ras/p44/p42 pathway, as monitored by the analysis of ERK1/2 phosphorylation (Fig. 6). These results contrast with prior studies that assigned an inhibitory role to caveolin-1 in activation of the p44/p42 MAP-kinase pathway (18). siRNA-mediated

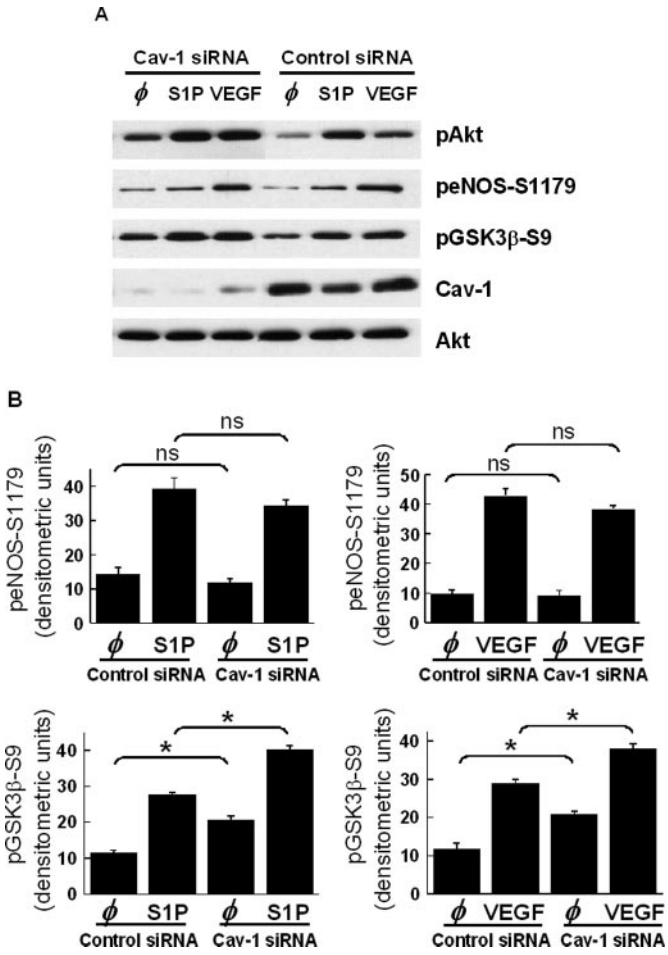


FIG. 9. **Caveolin-1 knockdown differentially affects phosphorylation of Akt substrates.** *A*, BAEC transfected with caveolin-1 siRNA or control siRNA were stimulated with S1P (100 nM) or VEGF (20 ng/ml) for 5 min. After treatment, cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies directed against phospho-Akt, phospho-eNOS (Ser-1179), or phospho-GSK3$\beta$ (Ser-9). Caveolin-1 expression and equal loading of the samples were assayed by probing the membranes with caveolin-1- and Akt-specific antibodies, respectively. This figure shows the results of a representative experiment that was repeated independently five times with equivalent results. *B*, results of densitometric analyses of pooled data, plotting the degree of eNOS (Ser-1179) and GSK3$\beta$ (Ser-9) phosphorylation in caveolin-1 or control siRNA-transfected cells both under basal conditions and after treatment with S1P or VEGF. Each *data point* represents the mean ± S.E. derived from five independent experiments; under all conditions, the addition of either S1P or VEGF induced a significant increase in phosphorylation of eNOS and GSK3$\beta$ ($p < 0.001$). *, $p < 0.001$ for caveolin-1 *versus* control siRNA transfected cells (using ANOVA). *ns*, not significant; *ϕ*, vehicle.

silencing of a gene homologous to caveolin in *Caenorhabditis elegans* also yielded a phenotype consistent with hyperactivated Ras signaling (59). By contrast, a recent publication (14) reported that embryonic fibroblasts isolated from caveolin-1-null mice had no substantive differences in ERK1/2 phosphorylation levels relative to wild-type, whereas fibroblasts isolated from cardiac tissue in these mice showed up-regulation of the p42/44 MAP kinase cascade (60). Taken together, these results affirm the highly cell-specific nature of caveolin-modulated regulation of signaling pathways.

In contrast to the nominal effects of caveolin-1 knockdown on ERK1/2 responses in BAEC (Fig. 6), we found that siRNA-mediated caveolin-1 knockdown markedly increased basal as well as agonist-mediated phosphorylation of the protein kinase Akt (Figs. 7 and 8). These results suggest that caveolin-1 may tonically suppress some component(s) of the Akt pathway that

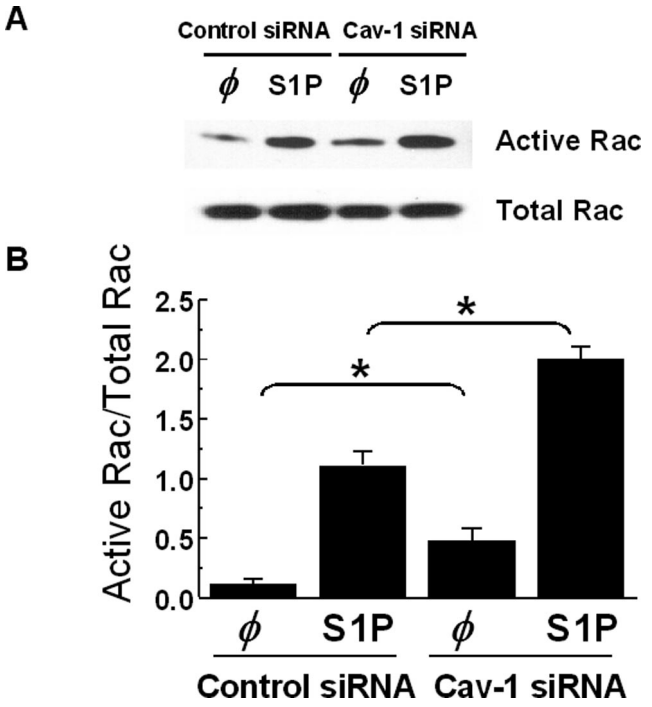


FIG. 10. **Caveolin-1 knockdown enhances Rac activity.** *A*, BAEC transfected with control or caveolin-1 siRNA were stimulated with S1P (100 nM) for 5 min. After agonist stimulation, Rac activity in the cell lysates was measured by pull-down of Rac-GTP using a glutathione *S*-transferase-p21 binding domain of the Rac effector molecule PAK-1. Precipitated Rac and total Rac in the cell lysates were quantified in immunoblots probed with a monoclonal antibody directed against Rac. *B*, densitometric analysis of pooled data, showing the ratio between active Rac and total Rac in caveolin-1 or control siRNA-transfected cells both under basal conditions and after treatment with S1P. Each *data point* represents the mean ± S.E. derived from five independent experiments; under all conditions, the addition of S1P induced a significant increase in Rac activity ($p < 0.001$). *, $p < 0.01$ for caveolin-1 *versus* control siRNA transfected cells (using unpaired *t test*). *ϕ,* vehicle.

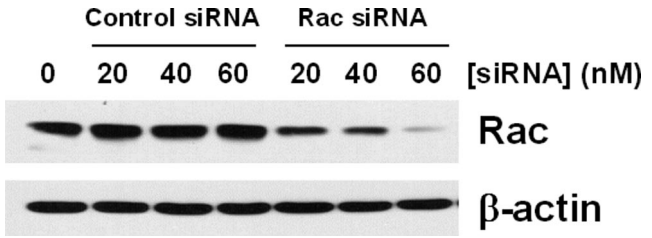


FIG. 11. **siRNA-mediated down-regulation of Rac expression in BAEC.** BAEC were transfected with the indicated concentrations of duplex siRNA targeted against Rac *(Rac siRNA)* or a random sequence (*Control siRNA*). 48 h after transfection, cells were harvested and lysed, and protein levels were analyzed in immunoblots probed with a Rac antibody or β-actin antibody, as shown.

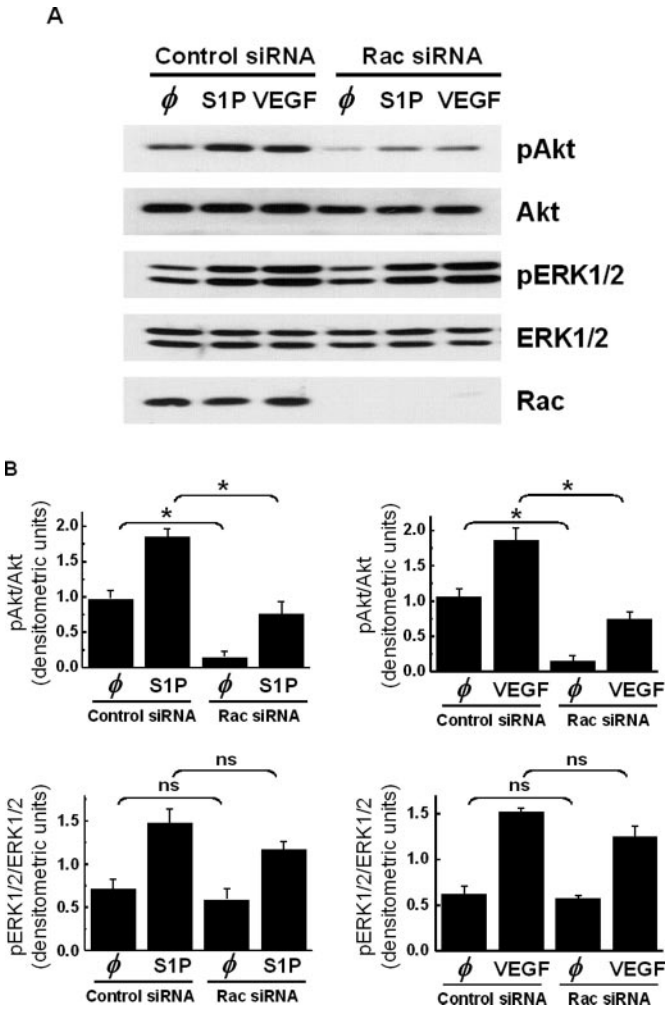


FIG. 12. **siRNA-mediated Rac knockdown down-regulates Akt phosphorylation in BAEC.** *A*, an immunoblot prepared from BAEC transfected with siRNA targeted against Rac or control siRNA and treated with S1P (100 nM) or VEGF (20 ng/ml) for 5 min. The cell lysates were resolved by SDS-PAGE and analyzed in immunoblots probed with antibodies against phospho-Akt, phospho-ERK1/2, Akt, and ERK1/2. Rac expression was analyzed by immunoblot with an anti-Rac antibody. *B*, results of densitometric analyses from pooled data, showing the level of Akt and ERK1/2 phosphorylation in Rac siRNA- or control siRNA-transfected cells under basal conditions and after treatment with S1P or VEGF. Each data point represents the mean ± S.E. derived from six independent experiments; under all conditions, the addition of either S1P or VEGF induced a significant increase in phosphorylation of Akt and ERK1/2 ($p < 0.01$). *, $p < 0.001$ for Rac *versus* control siRNA-transfected cells (determined by ANOVA). *ns*, not significant; *ϕ,* vehicle.

are activated after caveolin-1 knockdown and that are further enhanced after agonist stimulation. The effects of caveolin-1 siRNA on enhanced Akt phosphorylation could be blocked by wortmannin treatment, indicating that this response is dependent on PI3-kinase activation. We also observed (Fig. 9) that the caveolin-1 siRNA-mediated increase in Akt phosphorylation differentially affected the phosphorylation of two important substrates downstream from Akt kinase, specifically eNOS (42, 43) and GSK3β (44). The enhanced Akt phosphorylation in caveolin-1 siRNA-treated cells was associated with a significant increase in basal and agonist-induced phosphorylation of the Akt substrate GSK3β. By contrast, we found no significant differences between control and caveolin-1 siRNA-treated cells in the phosphorylation of eNOS at serine 1179, despite a robust increase in Akt phosphorylation. We note that although eNOS and GSK3β are each targets for Akt phosphorylation, other kinases have also been implicated in the phosphorylation of eNOS at serine 1179, including protein kinase A (61) and AMP-activated protein kinase (62). Likewise, GSK3β can also be phosphorylated by kinases other than Akt, including protein kinase A (63) and MAPK-activated protein kinase 1 (64). It remains possible that caveolin-1 knockdown affects the regulation of these other kinases or that cross-talk between these different signaling pathways could explain the differential effect of caveolin-1 knockdown on the phosphorylation pattern of these two Akt targets. Indeed, the role of caveolin-1 in the modulation of the PI3-kinase pathway is an area of active study. Li *et al.* (22) have recently reported that overexpression of caveolin-1 inhibits protein phosphatases PP1 and PP2A, leading to increased phosphorylation of Akt protein kinase in prostate cancer cells. Another study (21) reported that stable

overexpression of caveolin-1 leads to activation of the PI3-kinase pathway in HeLa cells. In contrast to these reports, Zundel *et al.* (20) found that overexpression of caveolin-1 inhibits the activity of PI3-kinase in Rat-1 fibroblasts. Taken together, these data suggest an important role for caveolin-1 in modulation of the PI3-kinase pathway, but these disparate findings also indicate that the effects of caveolin-1 on PI3-kinase pathways may differ importantly depending on the molecular context and cell type.

We observed that a fraction of Rac is targeted to caveolae/lipid raft domains in endothelial cells (Fig. 5), and siRNA-mediated caveolin-1 knockdown enhanced Rac activity in BAEC, both in the basal state and after S1P stimulation (Fig. 10). Several studies have reported that Rac can dynamically associate with caveolae/lipid rafts after agonist or integrin stimulation (39, 65), although a specific role for caveolin-1 in Rac activation has not been previously documented. These findings provide a biochemical rationale for our observations using confocal immunofluorescence microscopy (Fig. 2), in which caveolin-1 knockdown of endothelial cells was associated with an increase in lamellipodia and an enhancement of cortical actin distribution, both of which cellular features are characteristic of enhanced small GTPase Rac activity (46).

We also observed that siRNA-mediated down-regulation of caveolin-1 expression significantly enhanced S1P-induced migration of BAEC (Fig. 3). Pretreatment with the PI3-kinase inhibitor wortmannin attenuated the chemotactic response to S1P of control and caveolin-1 siRNA-treated BAEC, suggesting both that S1P-induced endothelial cell migration is dependent on PI3-kinase activation and that the augmentation of BAEC migration seen with caveolin-1 down-regulation is also modulated by PI3-K pathways. It is interesting that previous studies demonstrated that the small GTPase Rac can interact with the regulatory subunit of PI3-kinase, p85, an interaction that results in a marked enhancement of PI3-kinase activity (47, 66). Higuchi *et al.* (48) showed that overexpression of an active mutant of Rac was sufficient to induce Akt phosphorylation in fibroblasts, and PI3-kinase inhibitors abolished this effect, indicating that Rac induces Akt phosphorylation through PI3-kinase activation. Indeed, we observed that siRNA-mediated down-regulation of Rac expression significantly inhibited basal as well as S1P- and VEGF-induced Akt phosphorylation in BAEC (Fig. 12), indicating that Rac acts as an upstream modulator of the PI3-kinase/Akt pathway in endothelial cells.

Taken together, these studies suggest that caveolin-1 siRNA-mediated enhancement of Rac activity (Fig. 10) may represent a mechanism for the increase in Akt phosphorylation after caveolin-1 knockdown in endothelial cells (Figs. 6–9). siRNA-mediated down-regulation of caveolin-1 in endothelial cells may provide a useful experimental system to further delineate the interactions among Rac/PI3-kinase/Akt pathways as modulated by caveolin-1.

Our studies have validated the use of duplex siRNA transfection as a method to efficiently and selectively silence the expression of caveolin-1 and Rac in cultured endothelial cells and have yielded intriguing observations concerning the roles of caveolin-1 in protein targeting and kinase regulation. siRNA transfection may represent a promising tool to characterize pathways modulated by caveolin and other essential proteins involved in signal transduction in the vascular endothelium.

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