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Loss of Caveolin-1 Polarity Impedes Endothelial Cell Polarization and Directional Movement*

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The ability of a cell to move requires the asymmetrical organization of cellular activities. To investigate polarized cellular activity in moving endothelial cells, human endothelial cells were incubated in a Dunn chamber to allow migration toward vascular endothelial growth factor. Immunofluorescent staining with a specific antibody against caveolin-1 revealed that caveolin-1 was concentrated at the rear of moving cells. Similarly, monolayer scraping to induce random cell walk resulted in relocation of caveolin-1 to the cell rear. These results suggest that posterior polarization of caveolin-1 is a common feature both for chemotaxis and chemokinesis. Dual immunofluorescent labeling showed that, during cell spreading, caveolin-1 was compacted in the cell center and excluded from nascent focal contacts along the circular lamellipodium, as revealed by integrin β_1 and FAK staining. When cells were migrating, integrin β_1 and FAK appeared at polarized lamellipodia, whereas caveolin-1 was found at the posterior of moving cells. Notably, wherever caveolin-1 was polarized, there was a conspicuous absence of lamellipod protrusion. Transmission electron microscopy showed that caveolae, similar to their marker caveolin-1, were located at the cell center during cell spreading or at the cell rear during cell migration. In contrast to its unphosphorylated form, tyrosine-phosphorylated caveolin-1, upon fibronectin stimulation, was associated with the focal complex molecule phosphopaxillin along the lamellipodia of moving cells. Thus, unphosphorylated and phosphorylated caveolin-1 were located at opposite poles during cell migration. Importantly, loss of caveolin-1 polarity by targeted down-regulation of the protein prevented cell polarization and directional movement. Our present results suggest a potential role of caveolin polarity in lamellipod extension and cell migration.

Endothelial cell migration is a key step toward angiogenesis, a process that is required in a variety of physiological and pathological conditions, such as embryonic development, wound healing, tissue regeneration, and tumor growth and metastasis. A clear understanding of how endothelial cells sense chemoattractants, organize signaling asymmetry, and make a directional movement is of pivotal importance in the biology of normal cells, as well as tumor angiogenesis. Like other types of crawling cells, migrating endothelial cells acquire a series of spatially polarized features. The front of a migrating cell generates protrusive force associated with lamellipod or filopod protrusion coupled with the development of new cell adhesions to the extracellular substrates. Cell contractility is required to allow the retraction of the body and rear of the cell. Apart from the surface features, such as lamellipodia and microspikes, relatively little is known about the "directional sensing" machinery that orients locomotion machinery in endothelial cells.

Caveolae (also termed plasmalemmal vesicles) are specialized microdomains on the plasma membrane with a size of 50–100 nm (1). In addition to transcytosis and endocytosis, a good body of evidence has shown that caveolae compartmentalize and integrate signaling events at the cell surface (2–4). A variety of protein and lipid signaling molecules involved in VEGF[1] receptor and integrin-mediated signaling are concentrated in caveolae. These include VEGF-R2 (KDR), non-receptor tyrosine kinases (such as Src, Yes, and Fyn), PI 3-kinase, Rac1, Cdc42, and RhoA, and phosphatidylinositol (4–9). These observations suggest a potential role of caveolae in mediating signal transduction involved in cell migration.

The major structural proteins of caveolae are the caveolins. Four caveolins encoded by three different genes have been identified. Endothelial cells abundantly express caveolin-1 and -2 (but not caveolin-3, which is muscle-specific) (10). Caveolin-1 interacts with a number of signaling proteins, including Src family kinases, Gα subunits, H-Ras, protein kinase C, endothelial nitric-oxide synthase, PI 3-kinase, integrins, and epidermal growth factor receptor (11–14). In general, interaction between caveolin via the caveolin scaffolding domain and signaling proteins leads to inactivation of the target proteins (11). Thus, caveolin may function as an endogenous negative regulator of many signaling molecules. Given this view, one would predict that down-regulation of caveolins may lead to an increase in basal activity of signaling pathways and subsequent cellular activity, such as cell motility. In accordance with this, an attractive hypothesis would be that mere translocation of caveolin (*i.e.* caveolin polarization), without a substantial change in the expression level, would reinforce an inhibitory effect on one part of the cell (*i.e.* the cell rear) but release its inhibitory activity on the other side (*i.e.* the leading edge). This hypothesis is supported by recent studies showing caveolae and caveolin-1

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; TRITC, tetramethylrhodamine isothiocyanate; HUVEC, human umbilical vein endothelial cell; dsRNA, double-stranded RNA; siRNA, small interfering RNA; SH, Src homology.

asymmetry during cell migration (15–18). However, whether caveolin-1 polarity affects endothelial cell lamellipod protrusion and migration is not known.

In the present study, we demonstrated that caveolin-1 and caveolae were excluded from the leading edge and directed toward the rear of migrating cells. Interestingly, a subpopulation of caveolin-1 that was phosphorylated on tyrosine 14 specifically moved to focal adhesions at the leading edge of migrating cells. Importantly, loss of caveolin-1 polarity by targeted knockdown of the protein prevented endothelial cell polarization and impeded cell directional movement.

EXPERIMENTAL PROCEDURES

Materials—Reagents and other supplies were obtained from the following commercial sources: antibodies against caveolin-1, phosphocaveolin-1, and FAK from BD Biosciences (San Diego, CA). Phalloidin-TRITC and fibronectin were purchased from Sigma. Antibodies against integrin β_1, green fluorescent protein, and c-Myc were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat serum, fluorescein isothiocyanate, and Rhodamine Red-X-conjugated secondary antibodies were obtained from Jackson Immuno-Research Laboratories (West Grove, PA). Antibodies against paxillin, phosphopaxillin (pTyr-31), and phospho-FAK (pTyr-397) were obtained from BIOSOURCE International (Camarillo, CA). Slow-Fade reagent was purchased from Molecular Probes (Eugene, OR). The bicinchoninic acid protein assay kit was purchased from Pierce (Rockford, IL).

Cell Culture—Primary cultured endothelial cells from the human umbilical vein (HUVECs) were obtained as described previously (19) from consenting healthy, term patients according to institutional guidelines. HUVECs were grown in Medium 199 supplemented with 5% heat-inactivated human serum, 20% heat-inactivated newborn calf serum and endothelial cell growth supplement.

Construction of siRNA—The sequence (AAGAGCTTCCTGATTGAGATT) was selected as the targeting region of caveolin-1, which corresponds to nucleotides 403–423 of the coding region of human caveolin-1 (GenBank™ accession number BC009685). Caveolin-1 siRNA primers (5′-AATCTCAATCAGGAAGCTCTT-3′ and 5′-GAGCTTCCTGATTGAGATTTT-3′) were used for synthesis of double-stranded RNA (dsRNA) by Qiagen (Valencia, CA). Control siRNA was also obtained from Qiagen. The target sequence of the control siRNA was the DNA sequence AATTCTCCGAACGTGTCACGT, which contains a 16-base overlap with *Thermotoga maritimia* (GenBank™ accession number AE001709) section 21 of 136 of the complete genome, with no other BLAST matches. The level of caveolin-1 expression was determined by Western blot analysis and immunocytochemistry using specific antibody against caveolin-1. The specific down-regulation of caveolin-1 expression by caveolin-1 siRNA was assessed using control siRNA.

Transfection of Endothelial Cells with siRNA—Endothelial cells were seeded at a density of 6×10^4 cells/well in a 6-well plate 24 h prior to transfection. For each transfection, 1.0 μg of dsRNA was diluted into 200 μl of serum-free medium in a tube. In another tube, 6 μl of Oligofectamine (Invitrogen) were mixed with 54 μl of serum-free medium. The two solutions were combined, mixed gently, and incubated for 30 min at room temperature followed by the addition of 400 μl of serum-free medium. The cells were washed once with serum-free medium and incubated with dsRNA mixtures or Oligofectamine medium (mock transfection) for 4 h at 37 °C in a humidified CO_2 incubator. At the end of incubation, 2 ml of growth medium were added to each well, and the cells were incubated for 20 h. The medium was replaced with growth medium 24 h after transfection. Forty-eight h post-transfection (which resulted in an ~80% reduction in caveolin-1 level), the cells were used for a migration assay.

Scratch Motility Assay—Confluent endothelial cells were wounded by scraping of the monolayer with a 200-μl pipette tip (20). Cultures were washed once with phosphate-buffered saline and incubated with growth medium. The cells were incubated at 37 °C for 10 h to allow migration toward the gap and then fixed, permeabilized, and stained for caveolin-1.

Dunn Chamber Assay—Endothelial cells were seeded on fibronectin-coated coverslips and starved for 24 h prior to assay. To set up gradient experiments, both concentric wells of the chamber were filled with starvation medium (medium 199 with 0.5% fetal bovine serum), and a coverslip seeded with cells was inverted onto the chamber in an offset position leaving a narrow slit at one edge for refilling the outer well. The coverslip was sealed in place using hot wax mixture around all the edges except for the filling slit. The medium of the outer well was drained and replaced with medium containing 0.5% fetal bovine serum and 10 ng/ml VEGF. The slit was then sealed with hot wax mixture. When the inner circular well of the chamber was filled with control medium and the outer annular well with medium containing chemoattractant, a radially directed linear diffusion gradient was established in the diffusion gap within 10–30 min, which had a half-life of 10–30 h (21). For control experiments in which cells were subjected to uniform concentrations of chemoattractant, both wells were filled with medium containing VEGF (10 ng/ml). At the end of each migration assay, the coverslip was carefully removed from the Dunn chamber, and the cells were processed for immunocytochemistry analysis.

Immunocytochemistry—Endothelial cells were fixed with 2% paraformaldehyde for 20 min, permeabilized with 1% Triton X-100, blocked with 5% goat serum, and stained with specific antibody against caveolin-1 or other signaling molecules. Bound primary antibodies were detected using fluorescein- or rhodamine-conjugated secondary antibodies. The immunostained cells were mounted in the presence of Slow-Fade reagent. Immunostaining was visualized and photographed using a Zeiss LSM 510 confocal microscope.

Immunoblot Analysis—Proteins of the subcellular fractions were solubilized with SDS sample buffer containing 0.125 M Tris-HCl (pH 6.8), 5% (w/v) SDS, 2.5% (v/v) β-mercaptoethanol, 5% glycerol in double-distilled water. After boiling for 4 min, proteins were separated by SDS-PAGE (5–15% gradient gels) and electro-transferred to a nitrocellulose membrane for immunoblotting using enhanced chemiluminescence.

Transmission Electron Microscopy—Endothelial cells were either sparsely seeded onto gelatin-coated plates for 1 h or seeded near confluence, scratched multiply with a 200-μl pipette tip, and incubated to allow cell migration. The cells were then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer and scraped into a microcentrifuge tube. The cells were pelleted and post-fixed with osmium tetroxide. After fixation, the cells were infiltrated with a propylene oxide/epon mixture and stained with uranyl acetate and lead citrate. Thin sections of samples were examined under a JEOL 1220 transmission electron microscope. On randomly taken photographs, the number of caveolae at the leading edge and the cell center of spreading cells or at the leading edge and the cell posterior of migrating cells was determined.

RESULTS

Caveolin-1 Is Located at the Rear of Migrating Cells—Previous studies have shown that caveolin-1 displays polarization in migrating cells. For instance, caveolin-1 accumulated at the trailing edge of scratch-induced migrating bovine aortic endothelial cells or shear stress-stimulated bovine aortic endothelial cells (15, 16). On the other hand, caveolin-1 was concentrated at the leading edge of fibroblast growth factor-stimulated migrating bovine aortic endothelial cells (16). To assess whether the discrepancy of caveolin-1 polarization resulted from chemotactic gradient, we employed two kinds of cell migration systems: chemokinesis to measure random walk using monolayer denudation (also termed scratch motility assay) and chemotaxis to measure directional movement using the Dunn chamber. Six hours after scraping, the endothelial cells began to migrate into the wound gap. The closure of wound gaps took ~24 h. Immunofluorescent staining with a specific antibody against caveolin-1 showed that the majority of caveolin-1 was located at the rear of most moving cells (Fig. 1, *b* and *c*, *green arrowheads*) opposite the lamellipodium of the leading edge (Fig. 1*c*, *green arrows*). Similarly, when cells were exposed to a linear gradient of VEGF, caveolin-1 was concentrated at the rear or trailing edge of moving cells (Fig. 1*d*, *green arrowheads*). These results indicate that caveolin-1 polarization at the cell posterior is a common feature of both directional and random movements.

Mutual Exclusion Between Caveolin-1 and Focal Contacts at the Leading Edge—The migration-activated posterior relocation of caveolin-1 is intriguing and suggests that when cells are stimulated to migrate, caveolin-1 (along with caveolae; see Fig. 3) moves to the rear of the migrating cell as a mechanism to sequester it away from signaling proteins that direct cell motility at the leading edge. Here, we focused on nascent focal adhesions at the leading edge revealed by immunostaining with specific antibody against integrin β_1 and FAK. Human

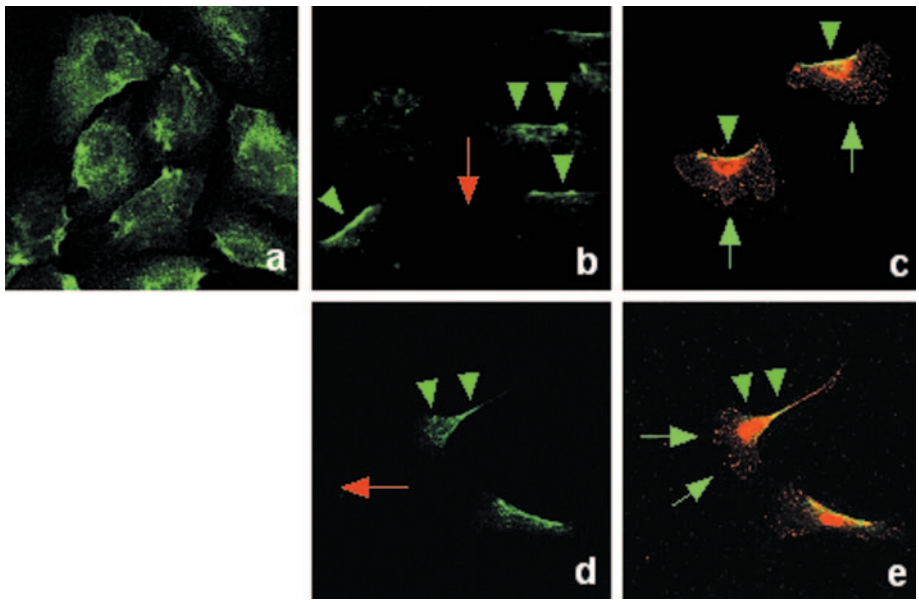


FIG. 1. **Caveolin-1 polarization during cell migration.** Confluent HUVECs were either cultured on coverslips (*a*) or wounded by scraping the monolayer with a pipette tip (*b*), incubated for 10 h to allow migration toward the gap (*red arrow* in *b*), and then fixed and immunostained with antibody for caveolin-1 (*a* and *b*) or dually stained with caveolin-1 (fluorescein isothiocyanate) and integrin β_1 (Rhodamine Red-X) (*c*) to reveal the leading edge of a migrating cell. Note that when cells migrated toward the wound gap, caveolin-1 was relocated to the rear of moving cells (*green arrowheads* in *b* and *c*) opposite the lamellipodia (*green arrows* in *c*). Dunn chamber assay (*d* and *e*). HUVECs were seeded on coverslips pre-coated with fibronectin and incubated at 37 °C to allow migration toward VEGF (*red arrow* in *d*) until they appeared in the bridge and outer well of the chamber (see "Experimental Procedures"). At that time, the coverslips were removed from the chamber, and the cells were fixed and subjected to dually immunofluorescent staining with specific antibody for caveolin-1 (fluorescein isothiocyanate, *d*) and integrin β_1 (Rhodamine Red-X, *e*). Note that caveolin-1 signal was again localized at the rear and trailing edge of moving cells (*green arrowheads* in *d* and *e*) opposite the leading edge (*green arrows* in *e*).

umbilical vein endothelial cells were seeded on fibronectin-coated coverslips. Within 1 h after seeding, most cells spread radially. As shown in Fig. 2, caveolin-1 was localized compactly in the center of the spreading cells (Fig. 2, *a* and *g*) and was excluded from nascent focal contacts along the circular lamellipodium revealed by FAK or integrin β_1 staining (Fig. 2, *b* and *h*). After incubation for several hours, focal contacts appeared in the polarized lamellipodia at the leading edge of migrating cells (Fig. 2, *e* and *k*). Caveolin-1 signal was now relocated at the rear of most moving cells (Fig. 2, *d* and *j*). These data confirm that caveolin-1 moves to the opposite pole of a migrating cell, compared with FAK and β_1 integrin at the leading edge.

Exclusion of Caveolae from Lamellipodia—Like many other terminally differentiated cells, such as adipocytes, fibroblasts, and skeletal muscle cells, endothelial cells possess a large number of caveolae (1, 22). Rapid freeze, deep etch images show that caveolae have a striated coat and contain the integral 22-kDa membrane protein, caveolin (23). The coat protein is a reliable marker for tracing caveolae trafficking in live cells (24). Based on our results described above demonstrating that caveolin-1 was centrally concentrated in spreading cells, we predicted that caveolae may be centrally localized as well. To assess the location of caveolae, endothelial cells were seeded for 1 h to allow spreading and then fixed and processed for electron microscopy analysis. As shown in Fig. 3, caveolae were localized abundantly at the cell center of spreading cells (Fig. 3*A*, *b*, *arrowheads*; Fig. 3*B*). Few, if any, caveolae were found at the lamellipod protrusion (Fig. 3*B*). When cells were migrating, caveolae, like their marker caveolin-1, were now concentrated at the cell rear (Fig. 3*A*, *d*, *arrowheads*; Fig. 3*C*), opposite the lamellipodium. In contrast, few, if any, caveolae were found at the leading edge of migrating cells (Fig. 3*A*, *e*; Fig. 3*C*). Again, these results indicate that caveolae, along with caveolin-1, were excluded from the leading edge of migrating cells.

Phosphocaveolin-1 Is Co-localized with Focal Complex Molecules at the Leading Edge of Migrating Cells—Caveolin is one of the major v-Src substrates in Rous sarcoma virus-transformed chicken embryo fibroblasts (25). Stimulation of A431 cells with EGF or NIH 3T3 cells with hyperosmotic stress leads to phosphorylation of caveolin-1 on tyrosine 14, and the phosphorylation events are associated with focal adhesions (26, 27). To assess whether phosphorylation of caveolin-1 occurred and where the phosphorylation took place during cell migration, endothelial cells were treated with fibronectin and dually stained with specific antibodies against phosphocaveolin-1 and either phosphopaxillin or caveolin-1. Dual immunolabeling revealed that phosphocaveolin-1 co-localized with the focal adhesion marker, phosphopaxillin, at the leading edge of migrating cells (Fig. 4, *a–c*), indicating that caveolin-1 was associated with focal complexes along the lamellipodium concomitant with tyrosine 14 phosphorylation. In contrast, a significant population of caveolin-1 that was not immunoreactive with Tyr-14 phosphospecific antibodies localized to the rear of migrating cells (Fig. 4, *e* and *f*). These data indicate that when tyrosine-phosphorylated, caveolin-1 associated with focal complex molecules at the leading edge of a migrating cell.

Targeted Knockdown of Caveolin-1 Impedes Cell Polarity—Our results demonstrated that caveolin-1 was directed to the posterior of either directionally or randomly moving cells, suggesting that caveolin-1 may play an important role in cell movement. We reasoned that during migration, caveolin-1 together with caveolae move to the rear of a migrating cell as a mechanism to sequester it away from signaling proteins that direct cell motility at the leading edge and that loss of caveolin-1 asymmetry by down-regulation of the protein would impede cell polarity and hence, cell migration. To test this hypothesis, we employed RNA interference to knock down caveolin-1 and examine its effect on cell polarity and migration. HUVECs were transfected with either caveolin-1 siRNA or control siRNA for 48 h and then seeded on fibronectin and incubated to allow migration. As shown in Fig. 5*C*, transfection with caveolin-1 siRNA specifically knocked down caveolin-1

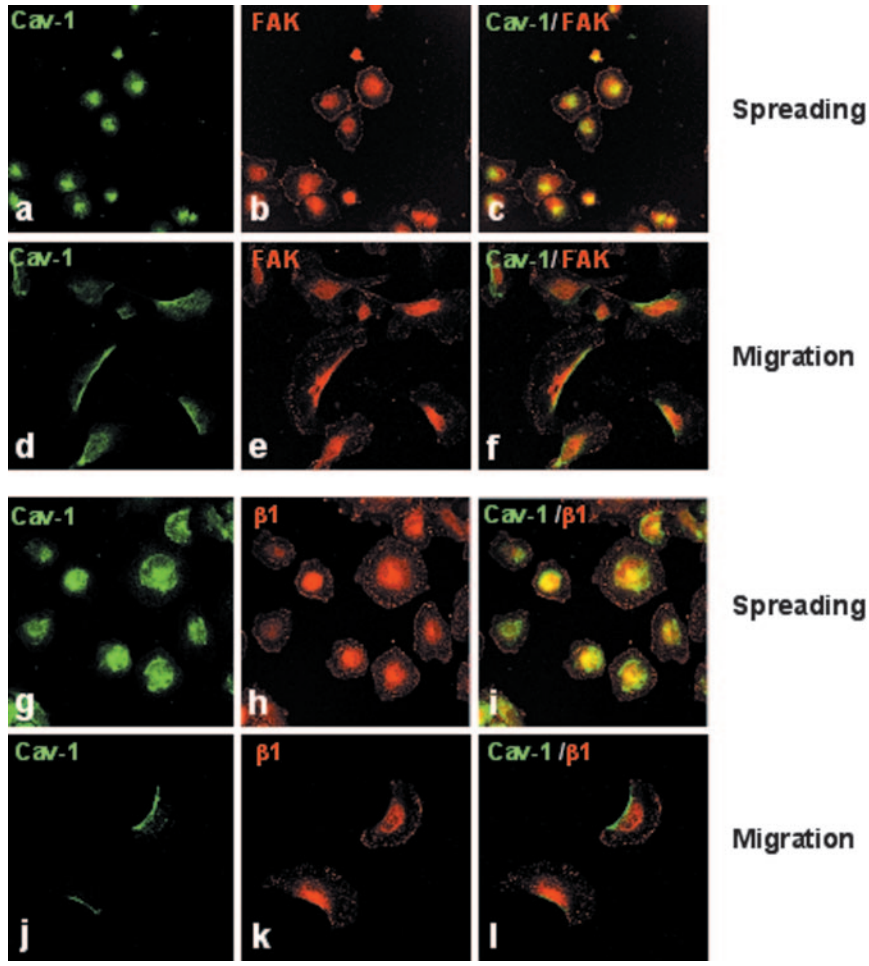


FIG. 2. **Mutual exclusion between caveolin-1 and focal contacts at the leading edge.** HUVECs were seeded on fibronectin-coated coverslips and incubated at 37 °C either for cell spreading assay or cell migration assay. After incubation, the cells were fixed, permeabilized, and subjected to dual immunofluorescent staining with caveolin-1 (*a*, *d*, *g*, and *j*) and either FAK (*b* and *e*) or integrin β_1 (*h* and *k*). Composite images (*c*, *f*, *i*, and *l*) resulting from the superimposition of caveolin-1 signal and FAK or integrin β_1 are shown. Note that during cell spreading, caveolin-1 was excluded from nascent focal contacts, revealed by FAK and integrin β_1 staining along the leading edge and located compactly in the cell center. When cells were migrating, focal contacts localized to one or sometimes two polarized lamellipodia, whereas caveolin-1 appeared at the body and rear of moving cells.

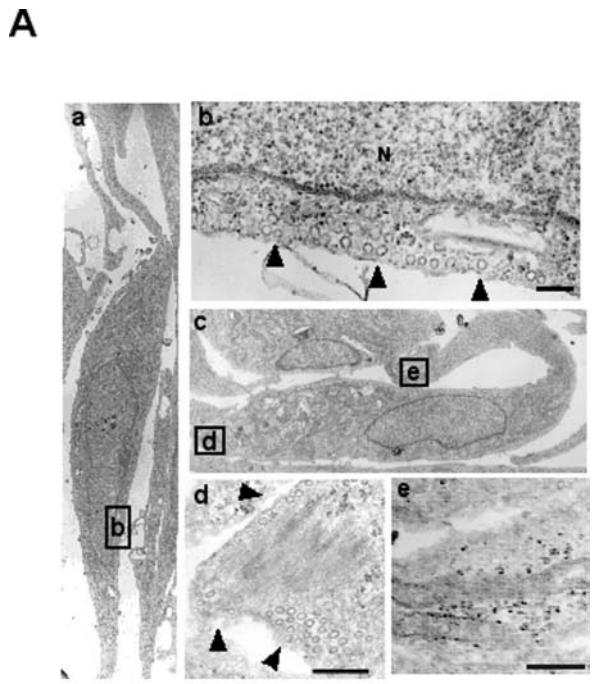
levels by >80%. Notably, knockdown of caveolin-1 dramatically impeded the ability of endothelial cells to polarize, which instead maintained a near-circular lamellipodium (Fig. 5*A*, *b*), whereas control siRNA- or mock-treated cells were able to polarize as normal (Fig. 5*A*, *a* and *c*). A comparison of Cav-1 siRNA-treated with control siRNA- or mock-treated cells revealed a significant reversal of the ratio of circular to polarized cells in the Cav-1 siRNA group, with caveolin-1 knockdown inhibiting the polarization of endothelial cells (Fig. 5*B*).

Knockdown of Caveolin-1 Inhibits Endothelial Cell Directional Movement—Given the nature of caveolin-1 as a scaffolding protein to organize and sequester signaling molecules, caveolin-1 may coordinate cellular activities between the leading edge and rear of a moving cell. In response to environmental stimulation, cells exclude caveolin-1 from the leading edge by an unknown mechanism and eliminate its inhibitory action on signaling molecules that are involved in lamellipod protrusion. At the same time, caveolin-1 concentrates and reinforces its inhibitory action at the posterior of a polarized cell. Hence, a cell may sense and move directionally by exclusion of caveolin-1 from the leading edge. In accordance with this, loss of caveolin polarity would inhibit directional cell movement. To test this hypothesis, HUVECs were transfected with caveolin-1 siRNA and subjected to chemotactic response to serum using a well characterized microchemotaxis chamber system (28). As shown in Fig. 6, loss of caveolin-1 polarity by knockdown of the protein dramatically reduced the number of migrating endothelial cells by >3-fold over mock or control siRNA-treated cells. Thus, our results indicate that loss of caveolin-1 asymmetry impeded endothelial cell directional movement.

DISCUSSION

We have demonstrated that caveolin and caveolae polarized regardless of VEGF-induced directional movement or monolayer scraping-induced random walk. Polarization was found at the rear of moving cells in both models. Notably, during cell spreading or migration, extension of the leading edge and protrusion of lamellipodia were not observed in the area with higher caveolin-1 signal. Although caveolin has an intimate relationship with the cytoskeleton, little is known about the function of the protein in cell migration. In the present study, we have shown that loss of caveolin polarity by knockdown of the protein dramatically inhibited cell polarization and impeded cell directional movement. Our results indicate an essential role of caveolin polarity in lamellipod protrusion and in orienting directional movement in endothelial cells. Fig. 7 depicts a model of cell polarization in which caveolin-1 is located at the rear of a moving cell, where it prevents lamellipod protrusion. Upon phosphorylation at tyrosine 14, caveolin-1 is released from caveolae and associated with focal adhesion sites at the leading edge, where it may mediate the recruitment of Csk and affect the formation of focal adhesions (29).

Our present results are consistent with previous reports demonstrating caveolin and caveolae polarization in bovine aortic endothelial cells in response to shear stress (15), monolayer scraping, or fibroblast growth factor stimulation (16). Our result of growth factor-induced caveolin relocation at the cell posterior stands in contrast to a recent report that caveolin is located to the leading edge of fibroblast growth factor-stimulated transmigrating bovine aortic endothelial cells (16). Interestingly, the leading edge accumulation of caveolin-1 requires



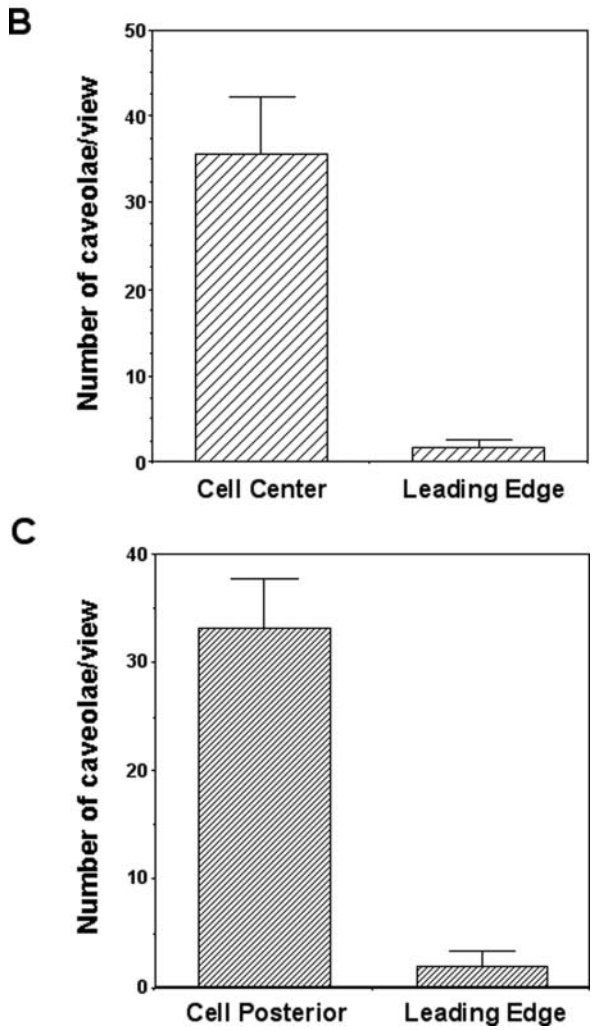


FIG. 3. **Exclusion of caveolae from lamellipodia.** Endothelial cells were seeded for 1 h to allow spreading (*a* in *A*), or a confluent monolayer was scraped to induce cell migration (*c* in *A*). After incubation, the cells were fixed and processed for transmission electron microscopy. During cell spreading, caveolae were centrally located (*arrowheads* in *b*) in proximity to the nucleus (*N*) and excluded from lamellipodia. When cells were migrating, caveolae were found to be concentrated in the rear of the cells (*arrowheads* in *d*) opposite the lamellipodia (*e*). *Scale bars*, 0.2 μm in *B*; 0.5 μm in *d* and *e*. The number of caveolae at the leading edge and cell center of spreading cells (*B*) and at the leading edge and cell posterior of migrating cells (*C*) was determined by counting caveolae in randomly taken photographs. Data are the means ± S.D. from ten photographs.

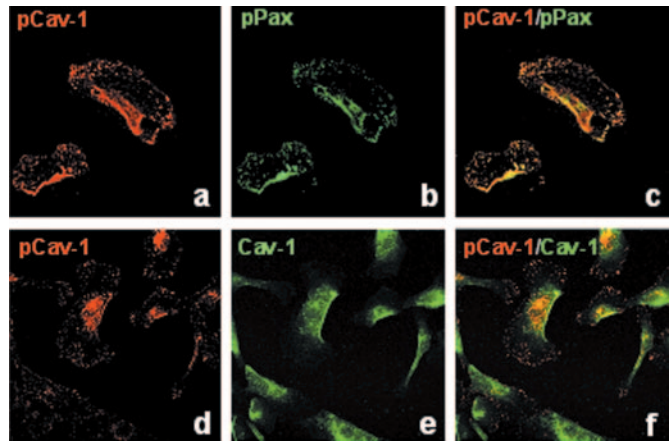


FIG. 4. **Co-localization of phosphocaveolin-1 with focal complex molecules at leading edge of migrating cells.** HUVECs were seeded on fibronectin-coated coverslips and incubated to allow migration. After incubation, the cells were fixed and stained with specific antibodies against phosphocaveolin-1 (*pCav-1*) (*a* and *d*), phosphopaxillin (*pPax*) (*b*), or Cav-1 (*e*). Composite images (*c* and *f*) resulting from the superimposition of pCav-1 signal and pPax or Cav-1 reveal a significant co-localization of pCav-1 with pPax (*c*) at focal complexes. In contrast, Cav-1 was concentrated in the body and rear of migrating cells opposite pCav-1 at the leading edge (*f*).

phosphorylation of the protein at tyrosine 14, which is consistent with our finding that tyrosine-phosphorylated endogenous caveolin-1 associated with focal adhesion molecules at the leading edge (see Fig. 4). This study did not determine whether the polarized endogenous caveolin-1 is tyrosine-phosphorylated; therefore, it is not clear whether they were observing forward relocation of non-phosphorylated or phosphorylated caveolin-1. Manes *et al.* (30) showed recently the relocation of lipid rafts to the leading edge of insulin-like growth factor-I-stimulated MCF-7 denocarcinoma cells. Because the study did not examine the location of caveolin-1, a marker of caveolae, it is unclear that the relocation of lipid rafts represents caveolae or non-caveolae rafts. Thus, caveolin and caveolae relocation depends on the modes of cell migration and is cell-type-specific. Nonetheless, the observation that different stimulations lead to caveolin and caveolae relocation suggests that it is a general mechanism for cells to spatially organize subcellular activities that direct cell motility.

The majority of caveolin-1 is found at the cell surface and associated with caveolae (23, 31). Some Golgi-associated caveolin-1 is in transit from its site of synthesis in the endoplasmic reticulum to the cell surface (32). The recycling of surface caveolin-1 through the Golgi apparatus involves the directional movement of the molecule from caveolae to the lumen of the endoplasmic reticulum and onto the Golgi apparatus (33, 34). One possible mechanism that directs caveolae and caveolin-1 polarization is that recycling caveolar vesicles accumulate at the cell rear during migration. Another possibility is that caveolin-1 and caveolae accumulate at the cell posterior as a result of differential movement of other organelles. The experimental observation that cell polarization signals are upstream and independent of those triggering cell motility suggests that

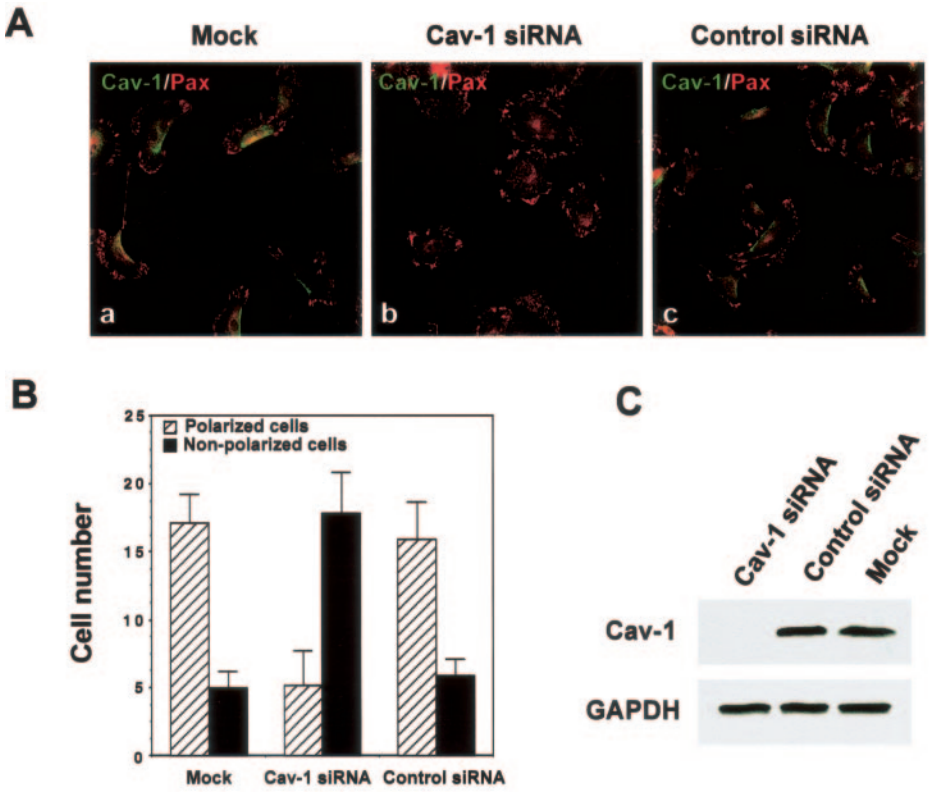


FIG. 5. **Knockdown of caveolin-1 prevents endothelial cell polarization.** HUVECs were transfected with either caveolin-1 siRNA or control siRNA, as described under "Experimental Procedures." *A*, forty-eight hours after transfection, HUVECs were seeded on fibronectin, incubated to allow migration, fixed, and then subjected to dual immunofluorescent staining with specific antibody for caveolin-1 (fluorescein isothiocyanate) or paxillin (Rhodamine Red-X). Note that mock- and control siRNA-treated cells were able to polarize (*a* and *c*). In contrast, knockdown of caveolin-1 impeded polarization of the cells (*b*), which instead displayed a near-circular lamellipodium. *B*, the effect of caveolin-1 knockdown on cell polarization was quantified by counting circular *versus* polarized cells from eight randomly selected views corresponding to each of the treatments. Data are the means ± S.D. *C*, caveolin-1 protein levels were specifically knocked down by the caveolin-1-specific siRNA.

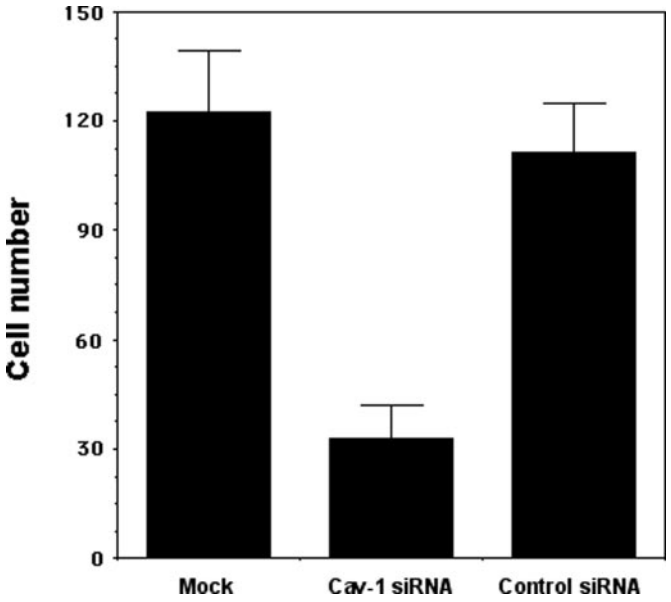


FIG. 6. **Knockdown of caveolin-1 inhibits endothelial cell directional movement.** HUVECs were transfected with either caveolin-1-specific or control siRNA or alternately were mock transfected. Forty-eight h after transfection, the cells were placed over a polycarbonate filter and allowed to migrate through 8-μm pores to an adjacent compartment in response to serum stimulation. After a 3-h incubation, the cells that migrated to the serum compartment were counted. Note that knockdown of caveolin-1 dramatically inhibited cell migration compared with control siRNA- or mock-treated cells. Data are means ± S.D.

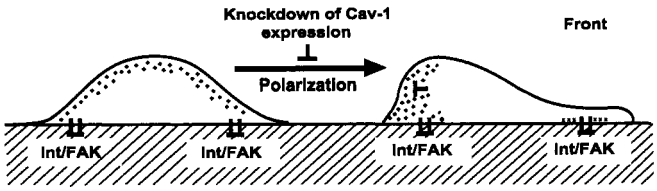


FIG. 7. **Proposed model demonstrating caveolin-1 polarity and control of lamellipod protrusion.** Caveolin-1, the integral membrane protein of caveolae, is believed to interact with signaling molecules. Immunofluorescent staining of a resting cell (*left*) with antibody for caveolin-1 reveals a punctate staining pattern at the cell surface (*small dots*, *left*). Upon chemotactic stimulation (*right*), caveolin-1 is excluded from the leading edge and relocates to the cell posterior (*small dots*, *right*) where it prevents lamellipod protrusion (*T bar*, *right*). In contrast, tyrosine-phosphorylated caveolin-1 is separated from caveolae and associated with focal complexes (*Int/FAK*) at the leading edge (*small crosses*, *right*). Thus, caveolin-1 polarity may serve to spatially organize cellular activity that mediates lamellipod protrusion. Loss of caveolin-1 polarity by targeted knockdown of the protein impedes cell polarization.

asymmetric caveolin redistribution is not the consequence of cell movement (35, 36).

Caveolin-1 was first identified as a major tyrosine-phosphorylated protein in v-Src-transformed chicken embryo fibroblasts (25). Microsequencing of Src-phosphorylated caveolin-1 revealed that phosphorylation occurs within the extreme N-terminal region of the full-length of caveolin-1 (37). Site-directed mutagenesis showed that tyrosine 14 is the principal substrate for Src kinase (37). Recent studies have shown that caveolin-1 undergoes phosphorylation at tyrosine 14 in response to a number of stimulations, such as insulin, EGF, and osmotic stress. Tyrosine-phosphorylated caveolin-1 provides a docking site recruiting SH2 domain-containing proteins, such as Grb7 and Csk, and augments EGF-stimulated cell migration (26, 29). Because Grb7 contains an SH2 domain and a phosphotyrosine-interacting region (38), it may function as a bridge linking phosphorylated caveolin-1 to other tyrosine-phosphorylated proteins, such as FAK (39). In the present study, we observed a rapid phosphorylation of caveolin-1 at tyrosine 14 upon fibronectin stimulation. Importantly, unlike the unphosphorylated caveolin-1, tyrosine-phosphorylated caveolin-1 was co-localized with focal complex molecules at the leading edge of migrating cells. The discovery that tyrosine-phosphorylated caveolin-1 and unphosphorylated caveolin-1 polarize at two opposing poles of moving cells is intriguing, although the mech-

anism underlying this is unclear. Therefore, during cell migration, phosphorylated caveolin-1 located at focal complexes along the leading edge might couple integrin to non-receptor tyrosine kinases, such as Src, Fyn, and FAK, whereas unphosphorylated inhibitory caveolin-1 is relocated to the cell rear.

Our present results demonstrated that loss of caveolin polarity impede endothelial cell polarity and directional movement and suggest that caveolin may play an important role in angiogenesis. This idea is supported by a variety of studies showing that caveolin-1 affects capillary formation. We and others have shown recently that antisense-mediated down-regulation of caveolin-1 inhibits capillary tubule formation (40, 41). Up-regulation of caveolin-1 in microvascular endothelial cells enhances capillary tubule formation via the caveolin-1 scaffolding domain (41). The importance of caveolin-1 in angiogenesis is further emphasized by a recent study demonstrating a reduced infiltration of blood vessels into fibroblast growth factor-supplemented Matrigel plugs in caveolin-1 knock-out mice (42). In the same mice, tumor weight, volume, and blood vessel density are reduced due to lack of caveolin-1 and caveolae (42).

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