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Identification of a Novel Domain at the N Terminus of Caveolin-1 That Controls Rear Polarization of the Protein and Caveolae Formation*

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When cells are migrating, caveolin-1, the principal protein component of caveolae, is excluded from the leading edge and polarized at the cell rear. The dynamic feature depends on a specific sequence motif that directs intracellular trafficking of the protein. Deletion mutation analysis revealed a putative polarization domain at the N terminus of caveolin-1, between amino acids 32–60. Alanine substitution identified a minimal polarizing domain at the N terminus of caveolin-1, between specific sequence motif that directs intracellular trafficking of the membrane. The dynamic feature depends on a component of caveolae, is excluded from the leading edge and polarized at the cell rear. The depolarization mutant in caveolin-1 null cells dramatically impeded caveolae formation. Furthermore, knockdown of caveolae formation by methyl-β-cyclodextrin failed to prevent wild-type caveolin-1 rear polarization. Importantly, genetic depletion of caveolin-1 led to disoriented migration, which can be rescued by full-length caveolin-1 but not the depolarization mutant, indicating a role of caveolin-1 polarity in chemotaxis. Thus, we have identified a sequence motif that is essential for caveolin-1 rear polarization and caveolae formation.

Caveolae are specific microdomains of the plasma membrane that were discovered more than 50 years ago (1). In endothelial cells, numerous vesicles appeared to derive from the uniformly flask-shaped invaginations, suggesting the endocytic potential of caveolae (2). Although the function of caveolae as transport vesicles mediating endocytosis and transcytosis remained obscure (3, 4), the identification, cloning, and characterization of caveolar coat proteins, caveolins, has increased our knowledge of caveolae, and a good body of evidence implicates caveolae in a specialized form of delivery of membrane components, extracellular ligands, bacterial toxins, and nonenveloped virus in several cell types (5–8). The caveolae-mediated endocytic pathway differs from that mediated by clathrin-coated pits. It is sensitive to protein kinase C inhibitors and cholesterol depletion (by filipin), and in some cells it is involved in the activation of protein tyrosine kinases (9). Phosphorylation at tyrosine 14 of caveolin-1 (Cav-1)2 may be required for the internalization of caveolae. The mechanism controlling caveolae trafficking remains unclear, but it apparently involves both microtubule and actin cytoskeletons (10). Surprisingly, using Cav-1 as a marker for caveolae, recent studies demonstrate that caveolae are rich in a variety of signaling molecules, with the implication that caveolae may function in the regulation of signal transduction. Given these views, an attractive hypothesis would be whether caveolae could carry signaling machinery to different locations of the cell to spatially organize signaling events. Indeed, Anderson and colleagues (11) have shown recently that concomitant with the relocation of caveolae, sites of Ca2+ wave initiation moved to the same location in migrating cells.

Three mammalian caveolins, i.e. Cav-1, -2, and -3, have been identified and characterized (12). Whereas Cav-1 and -2 are co-expressed in many cell types, Cav-3 is limited to muscle (13–15). Cloning and sequencing of Cav-1 cDNA showed that unlike clathrin, Cav-1 most likely is an integral membrane protein inserted into the membrane so that both the N and C termini of the protein are in the cytosol (16, 17). Biochemical studies have shown that caveolins interact with a variety of signaling molecules, and many of these caveolin-interacting proteins bear a common caveolin binding motif that is recognized by a 20 aa sequence (aa 82–101) proximal to the membrane insertion region. The simultaneous identification of Cav-1 as a caveolar membrane coat and as a component of detergent resistant trans-Golgi-derived vesicle (named VIP21, which stands for vesicular integral membrane protein of 21 kDa) implies that surface caveolin recycles between caveolae and the Golgi apparatus (18). Furthermore, caveolins are implicated in polarized vesicular traffic in epithelia (19) and in cholesterol...

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‡ The abbreviations used are: Cav-1, caveolin-1; aa, amino acid(s); ER, endoplasmic reticulum; HUVECs, human umbilical vein endothelial cells; MEFs, mouse embryonic fibroblasts; GFP, green fluorescent protein; N-CPD, N-terminal caveolin polarity domain; FBS, fetal bovine serum; MβCD, methyl-β-cyclodextrin; MES, 4-morpholineethanesulfonic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ad, adenovirus.
transport (20, 21). The intracellular trafficking apparently depends on a specific amino acid sequence in caveolin (22, 23).

We and others have shown recently that caveolae and Cav-1 are polarized in migrating endothelial cells (11, 24, 25). However, the mechanism underlying the asymmetrical distribution is not known. It is proposed that polarized caveolae and Cav-1 may play an important role in spatial organization of polarized signaling activity during cell migration, given the fact that caveolae are rich in signaling molecules and actively travel in cells upon stimulation. Indeed, depolarization of Cav-1 by targeted knockdown of the protein significantly impedes cell polarization and inhibits cell directional movement (24). In an attempt to identify specific sequence(s) or domain(s) that controls Cav-1 rear polarization in migrating cells, we generated mutant forms of Cav-1 fused to the reporter protein GFP and expressed them in cells lacking endogenous Cav-1. Here, we report the identification of a novel domain at the N terminus of Cav-1 that controls rear polarization of the protein as well as caveolae formation.

**EXPERIMENTAL PROCEDURES**

Antibodies and Reagents—pEGFP-N1 and monoclonal antibody for Cav-1 were purchased from BD Biosciences. Protein A/G plus-agarose immunoprecipitation reagent, polyclonal antibodies for GFP and Cav-1 (N-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-FLAG M2 antibody was purchased from Sigma. Rhodamine Red-X-conjugated secondary antibody was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). BJ5183-Red-X-conjugated secondary antibody was obtained from Jack-FLAG M2 antibody was purchased from Sigma. Rhodamine antibodies for GFP and Cav-1 (N-20) were purchased from BD Biosciences. Protein

Cell Culture—Primary mouse embryonic fibroblasts (MEFs) were obtained from day 13.5 mouse embryos. Pregnant mice were sacrificed with CO₂ asphyxiation. Embryos were decapitated, thoroughly minced, and trypsinized with 1 ml of 0.05% trypsin in 0.53 mM EDTA (Invitrogen) for 20 min at 37 °C. Ten ml of complete medium (Dulbecco’s modified Eagle’s medium) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) was used to inactivate the trypsin and resuspend the dissociated cells. Fibroblasts were plated on a 10-cm plate and cultured in a 37 °C, 5% CO₂ incubator. Early passages (passage <5) of primary MEFs were used for all experiments.

Human umbilical vein endothelial cells (HUVECs) were isolated from the human umbilical vein and cultured as described previously (24, 26). The cells were grown in MCD131 supplemented with 5% heat-inactivated human serum, 20% heat-inactivated newborn calf serum, 150 μg/ml endothelial cell growth supplement, 5 units/ml heparin sodium, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Plasmid Construction and Mutagenesis—The full-length cDNA encoding Cav-1 or mutants was fused in-frame to the N terminus of GFP. EcoRI and BamHI restriction sites were added to the 5′ and 3′ ends of murine Cav-1 cDNA by PCR using the

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A series of mutations in Cav-1 were generated by PCR from the cDNA of murine Cav-1 (see Fig. 1). The PCR products were subcloned into the EcoRI and BamHI sites of the pEGFP-N1 eukaryotic expression vector (Clontech). The orientation and sequence of Cav-1-GFP were verified by sequencing.

Cholesterol Depletion—Cholesterol depletion was performed as described previously (27). Briefly, wild-type MEFs were incubated with 0, 3, or 10 mM methyl-β-cyclodextrin (MβCD) in Dulbecco’s modified Eagle’s medium supplemented with 10% lipid-free FBS for 30 min at 37 °C. Then, the cells were replaced onto 1 μg/ml fibronectin-precoated coverglass to allow migration. After a 2-h incubation at 37 °C, cells were fixed for immunofluorescence co-staining with specific antibody for Cav-1 and paxillin or for transmission electron microscopy analysis. The percentage of Cav-1 polarization was calculated.

Adenovirus Production, Amplification, and Purification—FLAG-tagged normal and mutant cDNA of Cav-1 were cloned into pAdTrack-CMV, and the resultant constructs were digested with Pmel and electrotransformed into BJ5183-AD-1-competent cells for recombination. The recombinant adenoviral constructs were purified, digested with PstI, and transfected into AD293 cells to produce the recombinant adenovirus. After 7–10 days, the primary adenovirus was generated. The recombinant adenoviruses were named AdCav1–178, AdCav61–178, and AdCav46–50A. AdCav1–178 expressed FLAG-tagged full-length Cav-1 (Cav1–178-FLAG), and AdCav61–178 expressed FLAG-tagged N-terminal deletion of Cav-1 (Cav61–178-FLAG). AdCav46–50A expressed Cav-1 with alanine substitution of aa 46–50.

Upon reaching 90% confluency, AD293 cells were infected with the primary adenovirus. The cells were then incubated at 37 °C for 48 h. Following incubation, the cells were collected and suspended in 10 mM Tris-HCl (pH 7.9) buffer. Three freeze/thaw cycles were performed at −20 °C (until completely frozen)/37 °C (until fully thawed). The debris was pelleted, and supernatant was collected for purification. The adenovirus was purified by sequential cesium chloride gradient centrifugation. After purification, the virus was desalted and stored at −80 °C.

Chemotaxis Dunn Chamber Assay—Cav-1 null MEFs infected with AdCav1–178, AdCav61–178, or AdGFP were seeded on a glass coverslip coated with 0.2% gelatin and starved for 4 h prior to the assay. To set up gradient experiments, both centric wells of the chamber were filled with starvation medium (Dulbecco’s modified Eagle’s medium with 0.25% FBS), 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 medium of the outer well was drained and replaced with Dulbecco’s modified Eagle’s medium with 0.25% FBS, 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 medium of the outer well was drained and replaced with Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. For control experiments in which cells were subjected to uniform concentrations of chemotacticant, both wells were filled with medium containing 0.25% FBS.

The Horizon Method of Analyzing Chemotaxis—Cells migrating over the annular bridge of the Dunn chamber were recorded using a Zeiss LSM 510 laser-scanning confocal system with 10× objective. Differential interference contrast images were captured at 2-min intervals for a total of 6 h using Zeiss time-lapse software. Only cells migrating at least 20 μm were
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included in the calculation. The “horizon” distance was defined as the distance migrated from the starting position by 90% of the cells, a value determined arbitrarily to enable a highly stringent statistical test. Cell trajectories from three independent experiments were tracked manually and converted to a set of angular directions (28). These angles were displayed in a circular histogram where the length of each 18° segment represented the total number of cells with an average angle of migration falling within that particular interval. The Rayleigh test for unimodal clustering of directions was used to determine whether there was a significant chemotactic response (28). A uniform distribution (random cell motility) of data was assumed if the p value for the calculation was greater than 0.05. The mean direction is displayed as an arrow and the 95% confidence interval as a gray arc (Fig. 8A).

Immunofluorescence Microscopy—Cells expressing GFP-tagged normal or mutant forms of Cav-1 were plated on 0.2% gelatin-coated glass coverslips to allow migration, fixed with 2% paraformaldehyde in phosphate-buffered saline for 20 min at 22 °C, and then either mounted with Fluoromount-G (Southern Biotech Inc.) directly or permeabilized with 0.5% (v/v) Triton X-100 for 10 min at 22 °C. The permeabilized cells were blocked with 5% normal goat serum in phosphate-buffered saline for 1 h at 22 °C and incubated with primary antibody for 1 h followed by rhodamine Red-X-conjugated secondary antibody. Fluorescence images of the cells were acquired on an upright Carl Zeiss LSM 510 confocal microscope equipped with C-Apochromat 40×/1.2 W water-immersion objective using the 488 nm line of an argon laser and/or the 543 nm line of a HeNe laser at 25 °C. Cell borders were outlined from differential interference contrast images.

Images of 30–40 polarized cells expressing Cav-1–GFP were randomly recorded. Green fluorescence intensity in six regions (three at the cell front and three at the cell rear; see Fig. 2B) of each cell was measured using Image J software. Cav-1 depolarization was assumed if a ratio of rear-to-front fluorescence intensity was equal to or less than the cells expressing GFP. If a ratio of rear-to-front fluorescence intensity of a Cav-1 mutant was significantly higher than the cells expressing GFP but less than wild-type Cav-1 (Cav1–178–GFP), a partial polarization was assumed. We have shown that loss of Cav-1 polarity affects cell trafficking (22, 23, 30–32). To assess the trafficking of Cav-1 mutants by deletion mutations across the molecule, because this strategy had been used successfully to characterize domains for caveolar membrane targeting and intracellular trafficking, we reasoned that when cells are stimulated to migrate, Cav-1 moves to the rear of migrating cells in a sequence-specific fashion as a mechanism to sequester it away from signaling proteins that direct lamellipod protrusion. To determine the amino acid sequence that is necessary for Cav-1 polarization, we started by generating a series of caveolin-1 mutants by deletion mutations across the molecule, Cav1–156

Purification of Caveolin-rich Membrane Domains—Caveolin-rich membrane domains were isolated as described previously (29). In brief, confluent cells were scraped into sodium carbonate (pH 11.0) and homogenized. The resulting homogenate was mixed with the same volume of 90% sucrose in MES-buffered saline, which was overlaid by 35 and 5% sucrose in MES-buffered saline. The gradient was then centrifuged at 39,000 rpm for 16 h in an SW41 rotor, and then 12 fractions of 1 ml in each were collected, starting from the top of the gradient, for immunoblot analysis.

Transmission Electron Microscopy—Cav-1−/− MEFs were infected with or without AdCav1–178 or AdCav61–178. Twenty-four hours post-infection, cells were seeded onto gelatin-coated dishes, fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in phosphate buffer, and scraped into a microcentrifuge tube. The samples were post-fixed with osmium tetroxide and then embedded in epon. The sections were stained with uranyl acetate and lead citrate. Thin sections of samples were examined under a JEOL 1220 transmission electron microscope.

RESULTS

Deletion of aa 1–60 at the N Terminus Prevents Rear Polarization of Caveolin-1—Upon cell migration, Cav-1 is excluded from the leading edge and polarized to the cell rear. On the basis of previous extensive studies to identify domains that dictate caveolin intracellular trafficking, we reasoned that when cells are stimulated to migrate, Cav-1 moves to the rear of migrating cells in a sequence-specific fashion as a mechanism to sequester it away from signaling proteins that direct lamellipod protrusion. To determine the amino acid sequence that is necessary for Cav-1 polarization, we started by generating a series of caveolin-1 mutants by deletion mutations across the molecule, because this strategy had been used successfully to characterize domains for caveolar membrane targeting and intracellular trafficking (22, 23, 30–32). To assess the trafficking of Cav-1 mutants, we chose Cav-1−/− cells that were deficient in endogenous Cav-1 and would not sequester the mutants and keep them from depolarizing (see Fig. 3). The deletion mutants used in the present study include Cav32–178, Cav61–178, and Cav1–156 (Fig. 1). Previous studies have shown that deletion of aa 60–80 results in trapping the molecule in the endoplasmic reticulum (ER), whereas deletion of either aa 80–100 or 134–154 leads to co-localization with a Golgi marker (22). Thus, Cav-1 mutants that have been shown previously to sort improperly after expression were excluded from the present study. Wild-type and mutant Cav-1 generated as GFP fusion proteins were transiently expressed in Cav-1−/− MEFs and detected in a punctate pattern on the plasma membrane and, to a lesser extent, in the perinuclear regions (apparently the Golgi apparatus) in stationary cells (data not shown).

Our previous study showed that targeted knockdown of Cav-1 using Cav-1 siRNA (small interfering RNA) impeded human endothelial cell polarization by 3-fold and inhibited cell directional movement (24). This result was confirmed by our recent study demonstrating a dramatic decrease in the number of polarized mouse pulmonary endothelial cells from 32 ± 3.8% in wild-type cells to 16 ± 3.5% in Cav-1−/− cells (p < 0.01), suggesting that Cav-1 polarity plays an important role in cell
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Cavolin-1 | TM | C | Polarization
--- | --- | --- | ---
Cav1–178-GFP | | | Yes
Cav12–178-GFP | | | Yes
Cav16–178-GFP | | | No
Cav11–156-GFP | | | Partial
Cav4–46-GFP | | | Yes
Cav14–140-GFP | | | No
Cav4–51-GFP | | | No
Cav16–156-GFP | | | No

FIGURE 1. Schematic representation of wild-type and mutant Cav-1. Nine different Cav-1 constructs were used in this study. Cav-1 is divided into three domains. The N terminus (residues 1–101) and C terminus (residues 135–178) are separated by a hydrophobic transmembrane domain (TM; residues 102–134). Full-length and mutant Cav-1 were fused with GFP. The location of the alanine substitutions in each construct (Cav32–55AGFP to Cav51–55AGFP) is indicated by a black box. The ability of the constructs to polarize in migrating Cav-1−/− MEFs is indicated in the column to the right of each construct.

polarization. Although the number of polarized cells was dramatically reduced, some Cav-1 depolarized cells were able to polarize, suggesting that Cav-1 depolarization was not sufficient to completely block cell polarization. Thus, we expected that ectopic expression of full-length Cav-1 in Cav-1−/− cells would restore cell polarization and that expression of Cav-1 mutants would impede cell polarization and, subsequently, the polarity of the Cav-1 mutants. Therefore, in the present study, we focused only on the polarized subpopulation of cells to determine the polarity of Cav-1 mutants.

Consistent with previous observations (24, 25), Cav1–178−GFP polarized at the rear of migrating Cav-1−/− MEFs (Fig. 2A, a), suggesting that exogenously expressed Cav-1 behaves as endogenous protein and is able to polarize in Cav-1-deficient cells. Deletion of aa 1–31 retained Cav-1 rear polarity. In contrast, deletion of aa 1–60 (Cav61–178-GFP) prevented rear polarization of the protein, and instead signals of the mutant were detected at the leading edge (Fig. 2A, d), suggesting that aa 32–60 are necessary for Cav-1 polarization.

To assess whether the C-terminal domain plays a role in Cav-1 polarization, Cav-1−/− MEFs were transfected with a Cav-1 mutant lacking the final 22 amino acids (Cav1–156-GFP). As shown in Fig. 2A, c, the mutant polarized at the cell rear. In addition, weak signals were detected at the cell front, suggesting a partial polarization of the mutant.

To analyze the polarized Cav-1-GFP signal statistically in migrating cells, fluorescence intensity in six regions (three at the cell front and three at the cell rear; see Fig. 2B) was detected, and a ratio of rear-to-front fluorescence intensity was determined in each mutant (n > 30) (see “Experimental Procedures”) and compared with GFP only. Statistical analysis revealed that the ratio of rear-to-front fluorescence intensity in Cav1–178-GFP- or Cav32–178-GFP-expressing cells was 2.6- or 2.3-fold more than that in GFP, respectively (p < 0.0001; Table 1, Fig. 2C), suggesting that deletion of the first 31 aa at the N

FIGURE 2. Deletion of the N terminus of Cav-1 prevents polarization of the protein. A, Cav-1−/− MEFs were transfected with the constructs expressing Cav1–178-GFP (a), Cav32–178-GFP (b), Cav15–178-GFP (c), Cav61–178-GFP (d), or GFP alone (e). Twenty-four hours post-transfection, cells were seeded to allow migration, fixed with 2% paraformaldehyde, and examined by confocal fluorescence microscopy (a–c, cell borders are outlined by a white line). Note that Cav1–178-GFP (a) and Cav32–178-GFP (b) polarize in Cav-1−/− MEFs. In contrast, deletion of the first 60 amino acids at the N terminus (d) prevents the protein from polarization, and the mutant signal is detected at the leading edge. Scale bar, 20 μm. B and C, images of polarized cells expressing either full-length or mutant Cav-1 were randomly recorded, and the fluorescence intensity in six regions (three at the cell front (F1–F3) and three at the cell rear (R1–R3)) (B) was measured as described under “Experimental Procedures.” A ratio of rear-to-front fluorescence intensity in each mutant was determined (C). Data are the mean ± S.E. from three independent experiments. Statistical analysis is shown in Table 1. *, p < 0.01 compared with GFP only; **, p < 0.01 with full-length Cav-1 (Cav1–178-GFP). D, the number of Cav-1 polarized cells was determined by comparing the ratio of rear-to-front fluorescence intensity in each mutant with that of GFP. Polarization of a Cav-1 mutant was assumed if fluorescence intensity ratio of the mutant was higher than 2 S.D. of the mean of GFP. Data are the mean ± S.E. from three independent experiments. E, a parallel set of the cells transfected with the constructs described in A were lysed, and the lysates were subjected to SDS-PAGE and Western blotting with antibody for GFP and GAPDH.

Table 1 Student’s t test analysis of the fluorescence intensity of Cav-1 deletion mutants

| Construct     | p value compared with Cav1–178-GFP | p value compared with GFP |
|---------------|------------------------------------|--------------------------|
| Cav1–178-GFP  |                                    | 6.640 × 10−9             |
| Cav32–178-GFP | 0.276                              | 1.540 × 10−5             |
| Cav61–178-GFP | 8.858 × 10−9                       | 0.724                    |
| Cav1–156-GFP  | 2.016 × 10−4                       | 5.407 × 10−5             |
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terminus does not affect the polarity of the protein. In contrast, deletion of an additional 29 aa dramatically reduced the fluorescence intensity ratio to the level of GFP, suggesting loss of polarity (Fig. 2C). The fluorescence intensity ratio in Cav_{1–156}–GFP was 1.6-fold higher than GFP (p < 0.01) but 1.6-fold lower than Cav_{1–178}–GFP (p < 0.01), indicating partial polarization (Table 1; Fig. 2C). The effect of deletion mutation on Cav-1 polarization was not due to protein expression levels (Fig. 2E).

The number of Cav-1-polarized cells expressing each construct was determined by comparing the ratio of rear-to-front fluorescence intensity in each mutant with that of GFP. As described above, we focused only on the polarized subpopulation of the cells. Polarization of a Cav-1 mutant was assumed if fluorescence intensity ratio of the mutant was more than 2 S.D. of the mean of GFP. Fig. 2D shows that 79.5 ± 5.7% of morphologically polarized cells expressing full-length Cav-1 display Cav-1 rear polarization. In contrast, up to 8.8 ± 0.25% of polarized cells expressing Cav_{1–178}–GFP show rear polarization. The number of Cav-1 rear polarization cells is 44 ± 5.5% of polarized cells expressing the partial polarity mutant, Cav_{1–156}–GFP.

Cav_{61–178}–GFP Regains Polarity by Interacting with Endogenous Caveolin-1—Cav-1 exists as a large molecular mass in living cells, which is mediated by the oligomer domain, aa 61–101 (33). It is possible that expression of the depolarization mutant Cav_{61–178}–GFP would affect the localization of endogenous Cav-1 in a migrating cell. To test this hypothesis, we expressed Cav_{61–178}–GFP in either HUVECs or wild-type MEFs known to express Cav-1 and examined the localization of endogenous Cav-1 using polyclonal antibody for Cav-1 (the antibody recognizes an epitope at the N terminus of Cav-1 and does not react with our depolarization mutant). Fig. 3A shows that the depolarization mutant Cav-1 is co-localized with endogenous Cav-1, and interestingly, the mutant is polarized together with wild-type Cav-1 at the cell rear. This result suggests that the mutant may be trapped and co-translocated with endogenous Cav-1 to the rear of migrating cells.

To assess whether the mutant interacted with endogenous Cav-1, wild-type MEFs were transfected with or without the depolarization construct expressing Cav_{61–178}–GFP and subjected to immunoprecipitation with specific antibody for GFP. As shown in Fig. 3B, endogenous Cav-1 is co-precipitated with Cav_{61–178}–GFP (monoclonal antibody (clone 2297) recognizes an epitope within aa 61–71 (34), and reacts with the depolarization mutant, Cav_{61–178}–GFP). To confirm this result, we generated an adenovirus harboring FLAG-tagged mutant Cav-1 (Cav_{61–178}–FLAG). Wild-type MEFs were infected with the adenovirus and subjected to immunoprecipitation with an antibody specific for FLAG. Again, endogenous Cav-1 was detected in the immunoprecipitation complex (Fig. 3B), suggesting an interaction between the mutant and endogenous Cav-1.

Caveolin-1 is a principal protein component of caveolar membranes and resists solubilization in nonionic detergents at cold temperatures (35, 36). This physical property reflects enrichment of cholesterol and glycosphingolipids in caveolae (17). To determine whether Cav_{61–178} was tightly associated with lipid rafts, HUVECs were infected with AdCav_{61–178} and extracted with alkaline sodium carbonate, an agent that strips membranes of peripherally associated proteins, followed by an established sucrose density centrifugation method to purify caveolae microdomains (37, 38). Fig. 4 shows that Cav_{61–178} is co-fractionated with endogenous Cav-1 (fraction 5, top panel). To assess whether the association with caveole membranes resulted from its interaction with endogenous Cav-1, Cav-1/−/− MEFs were infected with either AdCav_{1–178} or AdCav_{61–178} and caveolae were isolated. We found that most of the mutant protein was recovered in fractions 5–7, indicating an integration of the mutant itself into lipid rafts (Fig. 4, lower panel).

Deletion of aa 1–60 Impedes Caveolae Formation—Cav-1 is a known critical factor required for biogenesis of caveolae. Cav-1 is a cholesterol-binding protein and may facilitate the concentration of a critical mass of cholesterol required for caveolae
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Figure 5. Deletion of aa 1–60 impedes caveolae formation. A, Cav-1−/− MEFs were infected with either AdCav1–178 (a–c) or AdCav61–178 (d and e) or were mock infected (f). Twenty-four hours post-infection, cells were fixed and processed for transmission electron microscopy. As expected, there was no caveolar structure in Cav-1−/− cells (f). In contrast, transduced expression of wild-type Cav-1 (d and e) resulted in caveolae formation (arrows). Interestingly, deletion of the first 60 aa led to a dramatic reduction in caveolae formation (arrows in a–c), b, c, and e are images at higher magnification of images a and d, respectively. Scale bars: 100 nm for b, c, and e; 2 μm for a, and f. B and C, images were randomly recorded; 90 (AdCav1–178-infected) or 198 (AdCav61–178-infected) cells were viewed. The number of cells that contained caveolae (B) and the number of caveolae in each cell (C) were counted. Data are the mean ± S.E. from three independent experiments. *, p < 0.05. D, 24 h post-infection, a parallel set of cells was lysed and subjected to SDS-PAGE and Western blotting with antibody for Cav-1. The same membrane was reprobed with antibody for GAPDH to show equal protein loading.

Identification of the Minimal Amino Acid Sequence Required for Caveolin-1 Polarization—Our results thus indicate that aa 32–60 are critical for the rear polarization of Cav-1. To identify the minimal sequence that is necessary for Cav-1 polarization, we generated a series of consecutive quadro- or penta-alanyl codon substitutions from aa 32 to 55 (Fig. 1) and examined the localization of the mutants by fluorescence microscopy (Fig. 6). Substitution of aa 32–40 with alanine (Cav32–35A-GFP, Cav36–40A-GFP) did not affect Cav-1 polarity (Fig. 6A, a and b). Substitution of aa 41–45 with alanine (Cav41–45A-GFP) led to partial polarization of Cav-1 (c in Fig. 6A). In clear contrast, replacement of aa 46–55 with alanine (Cav46–50A-GFP, Cav51–55A-GFP) prevented the rear polarization of Cav-1 (Fig. 6A, d and e).

Statistical analysis revealed that the ratio of rear-to-front fluorescence intensity in Cav46–50A-GFP- and Cav51–55A-GFP-expressing cells was similar to GFP but was reduced by 3-fold compared with full-length Cav-1 (Cav1–178-GFP) (p < 0.0001) (Table 2; Fig. 6B). The fluorescence intensity in Cav41–45A-GFP was about 1.5-fold higher than GFP (p < 0.05) but 2-fold lower than Cav1–178-GFP (p < 0.0001), indicating partial polarization (Table 2; Fig. 6B). Thus, we have identified a sequence motif in Cav-1, 46TKEIDLVNRD55, that is essential for the rear polarization of the protein in migrating cells. The effect of alanine invagination. Overexpression of Cav-1 in a lymphocytic cell line that does not endogenously express the protein and lacks caveolae is sufficient to induce the formation of caveolae (39). Genetic ablation of Cav-1 generates animals that lack caveolae (40, 41).

Upon the discovery of their abundance in endothelium, caveolae have been postulated as vesicular carriers mediating endocytosis and transcytosis (7, 42). Indeed, caveolae have the molecular transport machinery for vesicle budding, docking, and fusion (43). Thus, at least in endothelium, caveolae may integrate signaling events with vesicular transport (44). We and others have shown that concomitant with Cav-1 polarization, caveolae move to cell posterior as well (24, 25), suggesting that caveolae may function as transport cargos carrying Cav-1 to cell rear. To test whether aa 1–60 of Cav-1 affect caveolae formation and whether caveolae are depolarized in cells expressing the depolarization mutant, Cav-1−/− MEFs were infected with adenovirus harboring either wild-type (AdCav1–178) or the mutant Cav-1 (AdCav61–178) and processed for transmission electron microscopy. Fig. 5A shows that expression of wild-type Cav-1 induces caveolae formation, which localizes abundantly at the rear of a migrating cell as we have demonstrated previously (24). Surprisingly, expression of the depolarization mutant Cav-1 dramatically decreased the number of cells that were induced to form caveolae, by more than 12-fold (Fig. 5B). Furthermore, the number of caveolae in each cell was dramatically decreased by up to 4-fold (Fig. 5C). The impairment of caveolae formation did not result from protein expression levels (Fig. 5D). Because few caveolae were formed by the depolarization mutant, we were not able to determine whether caveolae were polarized in these cells.
mutant was assumed if the fluorescence intensity ratio of the
was determined as described above. Polarization of a Cav-1
expression levels (Fig. 6 for each construct) were randomly recorded. Fluorescence intensity in six
regions was measured, and a ratio of rear-to-front fluorescence intensity in
Amino acids 46–55 are required for Cav-1 rear polarization. A, Cav-1−/− MEFs were transfacted with constructs with a series of con-
secutive quatra- or penta-alanine substitutions between aa 32 and 35 of
Cav-1. Twenty-four hours post-transfection, cells were seeded to allow
migration, fixed, and examined by confocal fluorescence microscopy (a, b, and f, cell borders are outlined in white). Note that substitution of amino
cacids 46–50 or 51–55 (d and e) with alanine prevents Cav-1 polarization. Scale bar, 20 μm. B, images of polarized cells expressing Cav-1 mutants (n = 30 for each construct) were randomly recorded. Fluorescence intensity in six
regions was measured, and a ratio of rear-to-front fluorescence intensity in
each mutant was determined as described in the legend to Fig. 2B. Data are the mean ± S.E. from three independent experiments. Statistical analysis is
shown in Table 2. *, p < 0.05 compared with GFP only; **, p < 0.01 with
full-length Cav-1 (Cav1–178-GFP). C, the number of Cav-1 polarized cells was determined as described in the legend to Fig. 2. Data are the mean ± S.E. from three independent experiments. D, a parallel set of the cells transfacted with the
constructs as in A was lysed, and the lysates were subjected to SDS-PAGE
and Western blotting with antibody for GFP and GAPDH. E, Cav-1−/− MEFs were infected with either AdCav1–178 or AdCav46–50A. Twenty-four hours post-infection, cells were fixed and processed for transmission electron
microscopy. Images were randomly recorded; 40 (AdCav1–178-infected) or 68
(AdCav46–50A-infected) cells were viewed. The number of cells that contained
caveolae (a) and the number of caveolae in each cell (b) were counted. Data are the mean ± S.E. from two independent experiments. *, p < 0.05. Twenty-
four hours post-infection, a parallel set of cells was lysed and subjected to
SDS-PAGE and Western blotting with antibody for Cav-1 (c). The same mem-
brane was reprobed with antibody for GAPDH to show equal protein loading.

The number of Cav-1-polarized cells expressing each construct was determined as described above. Polarization of a Cav-1
mutant was assumed if the fluorescence intensity ratio of the

| Construct          | μ value compared with Cav1–178-GFP | μ value compared with GFP |
|--------------------|-------------------------------------|--------------------------|
| Cav32–35A-GFP      | 0.226                               | 3.215 × 10⁻⁴             |
| Cav36–40A-GFP      | 0.203                               | 8.150 × 10⁻⁴             |
| Cav41–45A-GFP      | 3.799 × 10⁻⁵                        | 0.016                    |
| Cav46–50A-GFP      | 2.629 × 10⁻⁸                        | 0.153                    |
| Cav51–55A-GFP      | 3.935 × 10⁻⁹                        | 0.392                    |

A genetic model of Cav-1 depolarization and was used to test
their ability to migrate directionally toward chemoattractant
(10% FBS) using a well characterized chemotaxis Dunn cham-
ber (28). In this system, cells were viewed directly and recorded
over a 6-h period by a live cell time-lapse imaging system. Only
cells migrating at least 20 μm (the “horizon” distance) were
included in the calculation (see “Experimental Procedures”). Cell trajectories from three independent experiments were
tracked manually, converted to a set of angular directions, and

Student’s t test analysis of the fluorescence intensity of alanine
replacement mutants

Identification of Cav-1 Polarization Domain

The observation that mutation of aa 46–50 coincidentally blocked
Cav-1 polarization as well as caveole formation is interesting
and suggests that: 1) Cav-1 polarity is caveolae-dependent, or 2)
the sequence motif is required both for Cav-1 polarization and
caveole formation. To test these hypotheses, we employed an
alternative model by treating wild-type MEFs with MβCD to
reduce cellular cholesterol level, which is known to block
caveole formation (45) and disperse Cav-1 in the plasma mem-
branes (27). In the absence of MβCD, more than 50% of the
MEFs examined showed caveole structure, and the number of
caveole in those cells was about 14 caveole/cell. Treatment of
the cells with 10 mM MβCD dramatically suppressed the number
of cells containing caveole by more than 3.5-fold (Fig. 7A, b). The impairment of caveole formation did not result from protein expression levels (Fig. 6E, c). Thus, these
results suggest that aa 46–50 play important role in Cav-1
polarity and caveole formation.

Loss of Caveoles Fails to Prevent Cav-1 Polarization—The alternative model by treating wild-type MEFs with MβCD to
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the cells with 10 mM MβCD dramatically suppressed the number
of cells containing caveole by more than 3.5-fold (Fig. 7A, b). The loss of caveole formation failed to prevent Cav-1 rear polarization (Fig. 7B and C). Thus, these results suggest that a caveole-

Caveoline–Polarity Plays an Important Role in Cell Directional Movement—The alternative model by treating wild-type MEFs with MβCD to
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of cells containing caveole by more than 3.5-fold (Fig. 7A, b). The loss of caveole formation failed to prevent Cav-1 rear polarization (Fig. 7B and C). Thus, these results suggest that a caveole-

substitution on Cav-1 polarization was not due to protein
expression levels (Fig. 6D).

To assess whether the alanine substitution mutants affect
caveole formation, Cav-1−/− MEFs were infected with ade-
ovirus harboring either wild-type (AdCav1–178) or an alan-
ine substitution of aa 46–50 mutant (AdCav46–50A) and
processes for transmission electron microscopy. As shown in
Fig. 6E, substitution of aa 46–50 with alanine dramatically decreased (by 5-fold) the number of cells that were induced

to form caveole (Fig. 6E, a). Furthermore, the number of
caveole in each cell was dramatically decreased by 3.5-fold
(Fig. 6E, b). The impairment of caveole formation did not result from protein expression levels (Fig. 6E, c). Thus, these
results suggest that aa 46–50 play important role in Cav-1
polarity and caveole formation.

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TABLE 2
Student’s t test analysis of the fluorescence intensity of alanine
replacement mutants

Loss of Caveoles Fails to Prevent Cav-1 Polarization—The alternative model by treating wild-type MEFs with MβCD to
reduce cellular cholesterol level, which is known to block

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Caveoline–Polarity Plays an Important Role in Cell Directional Movement—The alternative model by treating wild-type MEFs with MβCD to
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substitution on Cav-1 polarization was not due to protein
expression levels (Fig. 6D).

The number of Cav-1-polarized cells expressing each construct was determined as described above. Polarization of a Cav-1
mutant was assumed if the fluorescence intensity ratio of the

| Construct          | μ value compared with Cav1–178-GFP | μ value compared with GFP |
|--------------------|-------------------------------------|--------------------------|
| Cav32–35A-GFP      | 0.226                               | 3.215 × 10⁻⁴             |
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| Cav46–50A-GFP      | 2.629 × 10⁻⁸                        | 0.153                    |
| Cav51–55A-GFP      | 3.935 × 10⁻⁹                        | 0.392                    |
chemotaxis, we infected Cav-1-deficient cells demonstrated disoriented migration (Fig. 8A). The number of cells containing caveolae and the number of caveolae in those cells were counted. Note that MJCD treatment dramatically reduces caveolae numbers. Data are the mean ± S.D. *, p < 0.05. B and C, after treatment with 0 (a), 3 (b), or 10 (c) mM MJCD, wild-type MEFs were fixed and stained with antibody for Cav-1 (8). Dotted lines indicate cell borders. Images were randomly recorded, and the number of Cav-1 polarized cells was counted (C). Note that knockdown of caveolae by MJCD does not affect Cav-1 polarization. Data are the mean ± S.D. D, a parallel set of the cells pretreated with MJCD as described in B was lysed, and cell lysates were subjected to SDS-PAGE and Western blot analysis with antibody for Cav-1.

displayed in a circular histogram (Fig. 8A). The majority of Cav-1-deficient cells demonstrated disoriented migration (Fig. 8A, a). To test the effect of the depolarization mutant Cav-1 on chemotaxis, we infected Cav-1−/− MEFs with the adenovirus harboring full-length Cav-1 (Cav1−178), the depolarization mutant Cav-1 (Cav61−178), or GFP only. The expression levels of the full-length and the depolarization mutant Cav-1 were similar to the level of wild-type MEFs (Fig. 8C). Interestingly, expression of full-length Cav-1 in the Cav-1−/− cells resulted in up-gradient directional migration (Fig. 8A, b). In contrast, expression of the depolarization mutant, Cav-1, was not able to rescue the disoriented migration of the Cav-1−/− cells (Fig. 8A, c), thus suggesting that the rear polarization of Cav-1 plays an important role in chemotaxis. Quantitative analysis shows that expression of full-length Cav-1 caused more than 80% cells to migrate toward serum, whereas cells expressing GFP or the depolarization mutant showed negligible chemotactic movement (Fig. 8B).

DISCUSSION

Cell migration involves the asymmetrical organization of plasma membrane features that are associated with the cytoskeleton and signaling molecules. We and other groups have recently shown that Cav-1 was specifically polarizes to the posterior of migrating cells (22, 24, 25). In the present study, we systematically identified and characterized a domain at the N terminus of Cav-1 that controls rear polarization of the protein. Previous studies have identified a number of functional elements in caveolin. These include identification of the caveolin scaffolding domain that interacts with multiple signaling molecules (46, 47); the segment that is involved in self-association to form oligomers (33); the Golgi and ER localization signals (22, 23); the caveolin-inhibitory domain that inhibits endothelial...

Identification of Cav-1 Polarization Domain

Figure 8. Caveolin-1 polarity plays an important role in cell directional movement. A, Cav-1−/− MEFs were infected with adenovirus expressing GFP only (AdGFP) (a), full-length Cav-1 (AdCav1−178) (b and d), or the depolarization mutant (AdCav61−178) (c). The directional migration of the cells was analyzed using a chemotaxis Dunn chamber. Cells were exposed to either a gradient from 0.25% to 10% FBS (a–c) or no gradient (0.25% FBS in both chambers) (d). The direction of the gradient is indicated by the arrow in e. The data are presented as a circular histogram in which the length of each 18° segment (as shown in f) represents the number of cells with an average angle of migration falling within that particular interval (see "Experimental Procedures"). The mean direction of chemotaxis is displayed as an arrow, and the 95% confidence interval as a gray arc. B, quantitative analysis of cell directional movement. The number of chemotactic cells was determined by counting the cells with net movement toward serum gradient (these include the cells in the segments between 0 and 90° and 270 and 360° (see A, panel e). Note that Cav-1−/− MEFS expressing GFP or the depolarization mutant show negligible chemotactic movement. Data are the mean ± S.D. *, p < 0.05 compared with GFP. C, Cav-1−/− MEFs were infected with AdCav1−178, AdCav61−178 or AdGFP. Twenty-four hours post-infection, cells were lysed, and the lysates were subjected to SDS-PAGE and immunoblotting with antibody for Cav-1. The same membrane was reprobed with antibody for GAPDH to show equal protein loading. WT, wild type.
Identification of Cav-1 Polarization Domain

Lial nitric-oxide synthase, c-Src, and protein kinase A (48, 49); and the N- and C-terminal membrane attachment domains (31, 32). These investigations have employed cell systems that express endogenous Cav-1. Evidence has shown that interaction between the transduced and endogenous Cav-1 may mask crucial trafficking information (Ref. 50 and our present study on HUVECs and wild-type MEFs). The success of the present study depended on employing a cell system that was genetically deficient in Cav-1. Using this system, we have identified a sequence motif, aa 46–55, that is essential for Cav-1 rear polarization and caveola formation in migrating cells.

The observations that deletion of aa 1–60 or mutation of aa 46–50 coincidently blocked Cav-1 polarization as well as caveola formation suggest that Cav-1 polarity may be caveola-dependent. This idea is consistent with caveolin functioning as transport cargos carrying Cav-1 to the rear of the cell. Thus, the mutation-induced Cav-1 depolarization may be indirect and may result from a loss of caveola structure. We tested this hypothesis by depletion of cholesterol to block caveola formation (45) and found that loss of caveola failed to prevent the polarization of endogenous full-length Cav-1. Taken together, our results suggest that Cav-1 polarity appears to be mediated by 1) caveolae and/or 2) a sequence motif at the N terminus of the protein.

Multiple sequence alignment of Cav-1, -2, and -3 shows that the sequence from aa 47 to 56 is 100% identical between Cav-1 and Cav-3 (16), suggesting that this segment may mediate common functions shared by the two proteins. Another N-terminal conserved sequence, designed as the signature domain, is aa 68–75, which contains the sorting signal (aa 66–70) responsible for exit from the ER (22). The sequence of aa 46–55 does not resemble any domain with known function. Thus, the conserved sequence represents a novel domain that dictates subcellular translocation of Cav-1. We propose the term N-CPD (N-terminal caveolin polarity domain). The N-terminal (aa 1–79) and C-terminal (122–178) tails of Cav-1 are believed to be unstructured hydrophilic tails that remain entirely cytosolic and are accessible for cytosolic protein interactions (16). Thus, N-CPD might interact with an unidentified factor(s) that controls Cav-1 polarization in migrating cells.

When expressed either in HUVECs or wild-type MEFs that express endogenous Cav-1, deletion of N-CPD failed to prevent the mutant Cav-1 from polarization. This was not surprising, given the nature of caveolin and caveolin self-interaction (via aa 61–101) to form homo-oligomers before incorporation into glycosphingolipid-rich membranes in the Golgi apparatus (19). Indeed, our present results show that the depolarization mutant co-localizes and interacts with endogenous Cav-1. These results are consistent with previous observations indicating that the deletion and alanine substitution mutants retain the same orientation with wild-type Cav-1, have relatively normal confirmation, and are not shunted into a degradation pathway (22). Furthermore, the mutant is tightly associated with lipid rafts. Surprisingly, deletion of aa 1–60 or mutation of aa 46–50 dramatically impeded caveola formation. This was likely not caused by the interruption of the binding between Cav-1 and cholesterol, because this mutant contains the putative free cholesterol-binding sequence (98VTKYWFYR103) (51). The results suggest that aa 46–50 may play a role in caveola formation.

In the present study, we found that when expressed in MEFs, the majority signals of our depolarization mutant, Cav61–178~GFP, which lacked the first 60 aa, localized in a punctate pattern on the plasma membrane and, to a lesser extent, in the Golgi apparatus. Similarly, Machleidt et al. (22) recently showed plasma membrane targeting of the same mutant that lacked the first 59 aa, Cav60–178 when it was expressed in CHO cells. A recent study showed that expression of a deletion mutant that lacked the first half of the N-terminal domain (aa 3–48) in Fischer rat thyroid cells resulted in the accumulation of the mutant in the Golgi apparatus, although it oligomerized normally and associated with lipid rafts (30). The difference apparently resulted from the cell types used for protein expression. For instance, Fischer rat thyroid cells are likely to exhibit more pronounced Golgi apparatus staining of ectopically expressed proteins, such as full-length Cav-1, than other types of the cells that express Cav-1 endogenously, suggesting that Fischer rat thyroid cells are more sensitive to the conformational changes of Cav-1 (52).

Mutageneisis and biochemical analysis suggest that Cav-1 is cycling between caveolae and intracellular compartments. The transport depends on critical elements in the molecule required for the proper targeting to intracellular compartments (22, 23, 53). One of the functions of Cav-1 trafficking appears to be the maintenance of cholesterol homeostasis (21). Cav-1 binds directly to and is tightly associated with free cholesterol (54), and it travels between the ER and caveola membrane as a cytosolic complex containing chaperone proteins and cholesterol (55). Another lipid molecule in caveolae tightly associated with Cav-1 is the glycosphingolipid GM1 (56). Thus, Cav-1, together with caveola (rich in cholesterol and glycosphingolipids), relocates to the cell posterior and provide a second function of caveolin trafficking, i.e. establishment of cell membrane polarity in migrating cells. The polarized cholesterol and glycosphingolipid are likely to cause membrane stiffness and reduced deformability at the cell rear (57). Indeed, our previous observation showed the absence of lamellipod protrusions where Cav-1 was polarized (24).

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