Palmitoylation of Caveolin-1 Is Required for Cholesterol Binding, Chaperone Complex Formation, and Rapid Transport of Cholesterol to Caveolae*

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Annette Uittenbogaard and Eric J. Smart‡
From the Department of Physiology, University of Kentucky Medical School, Lexington, Kentucky 40536

We previously demonstrated that a caveolin-chaperone complex transports newly synthesized cholesterol from the endoplasmic reticulum through the cytoplasm to caveolae. Caveolin-1 has a 33-amino acid hydrophobic domain and three sites of palmitoylation in proximity to the hydrophobic domain. In the present study, we hypothesized that palmitoylation of caveolin-1 is necessary for binding of cholesterol, formation of a caveolin-chaperone transport complex, and rapid, direct transport of cholesterol to caveolae. To test this hypothesis, four caveolin-1 constructs were generated that substituted an alanine for a cysteine at position 133, 143, or 156 or all three sites (triple mutant). These mutated caveolins and wild type caveolin-1 were stably expressed in the lymphoid cell line, L1210-JF, which does not express caveolin-1, does not form a caveolin-1 complex, and does not transport newly synthesized cholesterol to caveolae. All of the caveolins were expressed and the proteins localized to caveolae. Wild type caveolin-1, but not the cysteine mutants, was stably transported to caveolae. The triple mutant did not assemble into complete transport complexes (10–20 min) transported cholesterol to caveolae. Caveolin-1 has a 33-amino acid hydrophobic domain and three sites of palmitoylation in proximity to the hydrophobic domain. Palmitoylation of caveolin-1 is necessary for binding of cholesterol, formation of a caveolin-chaperone transport complex, and rapid transport of cholesterol to caveolae. We conclude that palmitoylation of caveolin is necessary for cholesterol binding and transport complex formation.

Caveolae are cholesterol/sphingomyelin-rich plasma membrane microdomains that are present in numerous cell types. Cholesterol is central to the structure and function of caveolae. The cytoplasmic surfaces of caveolae are studded with a characteristic coat structure that is disrupted and partially disassembled by cholesterol-binding drugs such as filipin and nystatin (3, 4). Cholesterol-binding drugs also cause invaginated caveolae to flatten within the plane of the membrane (4). Studies with cholesterol-depleted cells also demonstrated that the number of invaginated caveolae were dramatically reduced, whereas the number of invaginated caveolae returned to control levels when the cells were cholesterol replete (5). Proteins linked to the extracellular side of the plasma membrane by glycosylphosphatidylinositol can associate with caveolae in a cholesterol-dependent manner (5). In addition, palmitoylated proteins, such as endothelial nitric-oxide synthase, interact with caveolae in a cholesterol-dependent manner (6). Recently, Fielding and Fielding (7) demonstrated that low density lipoprotein-derived free cholesterol traffics to caveolae and that a caveola-associated lipoprotein, lipoprotein-derived cholesterol, is regulated by oxysterols. In addition, Fielding and Fielding (10) have demonstrated that scavenger receptor-mediated uptake of cholesterol esters, receptor-mediated transport of cholesterol esters, is cholesterol ester uptake. We recently demonstrated that cholesterol uptake by caveolae is cholesterol ester uptake.

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‡ To whom correspondence should be addressed: Dept. of Physiology, University of Kentucky, MS 508 C, 800 Rose St., Lexington, KY 40536. Tel.: 859-323-6412; Fax: 859-323-1070; E-mail: ejsmart@pop.uky.edu.

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Caveolin-1 Palmitoylation and Cholesterol Trafficking

Palmitoylation of caveolin is necessary for binding of cholesterol, formation of a caveolin-chaperone transport complex, and rapid, direct transport of cholesterol to caveolae. To test this hypothesis, we constructed palmitoylation-minus mutants of caveolin-1 and stably expressed these mutant proteins in caveolin-minus cells. We demonstrated that two of the palmitoylation sites in caveolae are necessary for cholesterol binding and formation of the caveolin-chaperone transport complex. These data provide a mechanistic basis for the interaction of caveolin and cholesterol.

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 medium, Geneticin, calf serum, glutamine, trypsin-EDTA, LipofectAMINE, and penicillin/streptomycin were from Life Technologies, Inc. The analytical silica gel thin-layer chromatography plates, heptane, petroleum ether, ethyl ether, acetic acid, and 2-propanol were from Fisher. [3H]Acetate (specific activity, 5.33 Ci/mmol) and [32P]Sphingomyelin (specific activity, 1250 Ci/mmol) were from DuPont. The Bradford assay kit was from Bio-Rad. Percoll was from Amersham Pharmacia Biotech. OptiPrep was from Life Technologies, Inc. The anti-caveolin IgG (caveolin-1) and anti-clathrin IgG were from Transduction Laboratories. Mouse IgG directed against the human transferrin receptor was supplied by Zymed Laboratories Inc. (San Francisco, CA). The anti-HSP90, anti-HSP56, anti-cyclophilin 40, and anti-caveolin A IgGs were from AbFrontier BioReagents. Caveolin was expressed using the Promega expression vector, pCI-Neo.

Buffers—Buffer A consisted of 20 mM Tris, pH 7.6, 157 mM NaCl, 0.5% (v/v) Tween 20. Buffer B consisted of 25 mM MES, pH 6.5, 0.15 M NaCl, 1% (v/v) Triton X-100, 60 mM octyl glucoside, 0.1% (w/v) SDS. Buffer C (5X sample buffer) was 0.31 M Tris, pH 6.8, 2.5% (w/v) SDS, 50% (v/v) glycerol, 0.125% (w/v) bromophenol blue.

Cell Culture—L1210-JF cells are a murine lymphocyte cell line that does not express caveolin (19). On day 0, 1 × 10⁷ cells were seeded into 100-mm dishes in RPMI 1640 medium plus 0.5% (v/v) glutamine, 25 mM HEPES, pH 7.4, and 10% (v/v) calf serum. Transfectants contained 300 mg/µl Geneticin. The cholesterol concentration changed to the indicated times. Transfection with Caveolin cDNA—cDNA was subcloned into a pCI-NEO vector (Promega) and transfected into a 100-mm dish. On the day of transfection, 20 µl of serum-free RPMI medium, LipofectAMINE, and penicillin/streptomycin were added to the DNA. The DNA was placed onto cells rinsed with serum-free RPMI media. The cells were incubated at 25 °C for 30 min. After the incubation, 6 ml of serum-free RPMI media was added. The cells were then gently mixed and incubated at 37 °C.

RESULTS

Expression and Localization of Caveolin-Palmitoylation Mutants—To examine the role of palmitoylation on caveolin function, each of the individual cysteine residues known to be palmitoylated (23) was converted to alanine (constructs 133, 143, and 156) or all of the cysteines were converted to alanines (triple mutant) (Fig. 1). Wild type caveolin cDNA and each mutant cDNA construct were confirmed by sequence analysis before being stably transfected into caveolin-negative, L1210-JF lymphocytes. Cells expressing each caveolin were processed to isolate caveolae, and the degree of caveolin expression and the subcellular location of each caveolin were determined by SDS-PAGE and immunoblot analysis (Fig. 2). Wild type caveolin, 133, 143, and 156 were expressed at comparable levels (±6%), whereas the triple mutant was expressed at 51 ± 10% of that of wild type caveolin. All of the caveolins were preferentially localized in the caveolae fraction (Fig. 2A, compare CM to PM). The non-caveola markers, clathrin and transferrin receptor, were excluded from the caveola fraction (Fig. 2B).

To confirm that palmitoylation of the mutant proteins was...
individual caveolin mutant proteins contained 30–66% of the radioactivity associated with each caveolin. To confirm the radioactivity associated with each caveolin was associated with the radiolabeled sterol fraction (data not shown). The extent of caveolin expression and the subcellular location of each caveolin were determined by SDS-PAGE and immunoblot analysis. An equal amount of protein (15 μg) from each subcellular fraction was resolved by SDS-PAGE and immunoblotted with IgGs for caveolin and cyclophilin. The precipitated material was then immunoprecipitated with caveolin IgG, and 50 μg of immunoprecipitated material was then immunoprecipitated with cyclophilin IgG (22). The precipitated material was extracted and the associated lipids resolved by thin-layer chromatography. All of the precipitated material was resolved by SDS-PAGE and immunoblotted with IgGs for caveolin, HSP56, cyclophilin 40, and cyclophilin A (22). To determine whether the mutant caveolins are capable of transporting cholesterol to caveolae, we used a temperature shift assay (22). Cholesterol can be synthesized at 14 °C in the endoplasmic reticulum, but little of the sterol can traffic through the cell (28). Shifting the temperature to 37 °C permits a bolus of labeled sterol to move through intracellular trafficking routes. Cells were chilled to 14 °C and then incubated with [3H]acetate for 2 h before adding excess unlabeled acetate and shifting the temperature to 37 °C for various times. The cells were then subfractionated and the amount of sterol in intracellular membranes (Fig. 6, open square) and caveolae (open circle) was determined by thin-layer chromatography and scintillation counting. As shown previously (19, 22), wild type caveolin transports radiolabeled sterol to caveolae within 10–20 min (Fig. 6A). Mutant 133 also transports similar

![Fig. 1. Caveolin cDNA constructs.](image)

![Fig. 2. Expression and localization of the caveolin mutants.](image)

![Fig. 3. Palmitoylation of caveolin.](image)

Alteration of Sterol with Caveolin—We next tested the ability of the mutant caveolins to bind to sterol. The cellular sterol pools were labeled by incubating cells for 16 h in the presence of [3H]acetate at 37 °C. The cells were lysed and the cytosol isolated by centrifugation at 250,000 × g for 1 h. The cytosolic pool of caveolin-1 was immunoprecipitated with caveolin IgG (22). The precipitated material was extracted and the associated lipids resolved by thin-layer chromatography. All of the precipitated material was resolved by SDS-PAGE and immunoblotted with IgGs for caveolin, HSP56, cyclophilin 40, and cyclophilin A. Wild type caveolin-1 and 133 coimmunoprecipitated HSP56, cyclophilin 40, and cyclophilin A. Mutant 143 coimmunoprecipitated HSP56 and cyclophilin 40 but not cyclophilin A. Wild type caveolin-1 and 133 coimmunoprecipitated HSP56 and cyclophilin 40 but not cyclophilin A. Mutant 156 coimmunoprecipitated cyclophilin 40 and cyclophilin A but not HSP56. The triple mutant only coimmunoprecipitated cyclophilin 40.

We previously demonstrated that the caveolin-chaperone transport complex will transport newly synthesized cholesterol from the endoplasmic reticulum to caveolin within 10–20 min (22). To determine whether the mutant caveolins are capable of transporting cholesterol to caveolae, we used a temperature shift assay (22). Cholesterol can be synthesized at 14 °C in the endoplasmic reticulum, but little of the sterol can traffic through the cell (28). Shifting the temperature to 37 °C permits a bolus of labeled sterol to move through intracellular trafficking routes. Cells were chilled to 14 °C and then incubated with [3H]acetate for 2 h before adding excess unlabeled acetate and shifting the temperature to 37 °C for various times. The cells were then subfractionated and the amount of sterol in intracellular membranes (Fig. 6, open square) and caveolae (open circle) was determined by thin-layer chromatography and scintillation counting. As shown previously (19, 22), wild type caveolin transports radiolabeled sterol to caveolae within 10–20 min (Fig. 6A). Mutant 133 also transports similar...
amounts of radiolabeled sterol to caveolae within 10–20 min (Fig. 6B). However, mutants 143 and 156 and the triple mutant did not translocate radiolabeled sterol to caveolae by 60 min (Fig. 6, C–E). Even after 4 h, mutants 143 and 156 and the triple mutant did not translocate radiolabeled sterol to caveolae (data not shown).

We next determined if the inability of the caveolin mutants to transport cholesterol affected the mass of cholesterol associated with caveolae. To determine this, caveolae were isolated from each mutant cell line, and the mass of cholesterol in each caveolae fraction was determined with a commercially available kit (9). Cells expressing wild type caveolin and 133 were highly enriched in cholesterol, whereas mutants 143 and 156 and the triple mutant were only slightly enriched in cholesterol (Fig. 7).

**DISCUSSION**

Caveolin has an unusually long hydrophobic region of approximately 33 amino acids followed by three cysteine residues that are known to be palmitoylated (23). We hypothesized that this hydrophobic region along with the palmitoylated cysteine residues could form a hydrophobic binding pocket to sequester and transport cholesterol through the cytosol. To test this hypothesis, caveolin mutants were generated that selectively removed one palmitoylation site each or all three sites. The present data suggest that the palmitoylation of caveolin plays...
a role in cholesterol binding and in the assembly of the chaperone complex. Cholesterol binding to caveolin requires palmitoylation at residues 143 and 156 but not at residue 133. Furthermore, cyclophilin 40 coimmunoprecipitated with caveolin regardless of the palmitoylation state of caveolin, suggesting that palmitoylation is not necessary for caveolin-cyclophilin 40 interactions. Lack of palmitoylation at position 133 did not affect the formation of a functional chaperone complex. However, lack of palmitoylation at 143 prevented the binding of cyclophilin A, whereas lack of palmitoylation at 156 prevented the binding of HSP56. In addition, the triple mutant and mutants 143 and 156 did not traffic cholesterol to caveolae.

We previously demonstrated a role for caveolin in intracellular cholesterol trafficking by using a lymphocyte cell line, L1210-JF, that does not express caveolin. The critical experiments directly followed the protocol of Kaplan and Simoni (28). In brief, cells are incubated with [3H]acetate at 14 °C to label L1210-JF, that does not express caveolin. The critical experiments demonstrated that the trypsin 143 and 156 did not traffic cholesterol to caveolae. In addition, the triple mutant and mutants 143 and 156 did not traffic cholesterol to caveolae.

The binding of HSP56. In addition, the triple mutant and mutants 143 and 156 did not traffic cholesterol to caveolae. The transport was not inhibited by brefeldin A, which suggested that this was cholesterol moving through the classic membrane secretory pathway and that L1210-JF cells expressing caveolin had a similar cholesterol trafficking kinetic profile. Cholesterol binding to caveolin requires palmitoylation at position 133. However, lack of palmitoylation at position 133 did not affect the formation of a functional chaperone complex. However, lack of palmitoylation at 143 prevented the binding of cyclophilin A, whereas lack of palmitoylation at 156 prevented the binding of HSP56. In addition, the triple mutant and mutants 143 and 156 did not traffic cholesterol to caveolae.

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