Cholesteryl Ester Is Transported from Caveolae to Internal Membranes as Part of a Caveolin-Annexin II Lipid-Protein Complex*

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Annette Uittenbogaard, William V. Everson, Sergey V. Matveev, and Eric J. Smart‡

From the Department of Physiology, University of Kentucky Medical School, Lexington, Kentucky 40536

We previously demonstrated that in Chinese hamster ovary cells scavenger receptor, class B, type I-dependent selective cholesteryl ester uptake occurs in caveolae. In the present study we hypothesized that cholesteryl ester is transported from caveolae through the cytosol to an internal membrane by a caveolin chaperone complex similar to the one we originally described for the transport of newly synthesized cholesterol. To test this hypothesis we incubated Chinese hamster ovary cells expressing scavenger receptor, class B, type I with [3H]cholesteryl ester-labeled high density lipoprotein, subfractionated the cells and looked for a cytosolic pool of [3H]cholesteryl ester. The radiolabeled sterol initially appeared in the caveola fraction, then in the cytosol, and finally in the internal membrane fraction. Caveolin IgG precipitated all of the [3H]cholesteryl ester associated with the cytosol. Co-immunoprecipitation studies demonstrated that in the presence of high density lipoprotein, but not low density lipoprotein-deficient serum, caveolin associated with four proteins: annexin II, cyclophilin 40, caveolin, and cyclophilin A. Caveolin acylation-deficient mutants were used to demonstrate that acylation of cysteine 133, was required for cholesterol to associate with caveolin and the rapid transport of cholesteryl esters out of caveolae. We conclude that association with caveolin and the rapid transport of cholesteryl esters from caveolae through the cytosol to an internal membrane by a caveolin chaperone complex is the mechanism of cholesteryl ester transport.

Caveolae are plasma membrane domains found in most types of cells and are identified by the presence of a 22-kDa protein called caveolin. Caveolae play a pivotal role in the formation, structural integrity, and function of caveolae (1). Caveolin has multiple functions, but the function of caveolin relevant to the present studies is its role in the trafficking of intracellular sterol. Caveolin can directly bind to cholesterol (2, 3), and in an earlier study, we demonstrated that acylation of caveolin was required for the binding of cholesterol to caveolin (4). We speculated that the acylation of caveolin, which occurs adjacent to the hydrophobic membrane domain of caveolin, forms a binding pocket that sequesters cholesterol from the aqueous environment (4). These studies were extended to demonstrate that caveolin is part of a lipid-protein chaperone complex that transports newly synthesized cholesterol from the endoplasmic reticulum directly to caveolae (2). The lipid-protein complex consists of cholesterol, caveolin, heat shock protein 56 (HSP56), cyclophilin 40, and cyclophilin A. Acylation of caveolin at cysteine residues 143 and 156, but not at 133, was required for cholesterol to associate with caveolin (4) and for the association of the lipid-protein complex. The lipid-protein complex (cholesterol-cyclophilin) transported newly synthesized cholesteryl esters from caveolae to internal membranes where it remained for some time before the cholesterol was effluxed to the plasma membrane (2) or was internalized and degraded (5, 6). Pharmacological inhibition of caveolin acylation with cyclophilin A or inhibition of newly synthesized caveolin with the cyclin-dependent kinase inhibitor rapamycin prevented the rapid translocation of newly synthesized cholesteryl esters from caveolae to internal membranes (7).

In CHO cells are able to internalize exogenous cholesteryl esters, but not cholesteryl esters from HDL. Internalization and degradation of HDL in CHO cells with SR-BI is a physiological receptor for HDL that facilitates the selective uptake of HDL cholesteryl esters (10). Hence (11) demonstrated that SR-BI is preferentially associated with caveolae in CHO cells, and we recently demonstrated that HDL-derived cholesteryl ester is initially transferred to caveolae (7). In the presence of HDL, caveolae rapidly saturates (~7 min) with cholesteryl ester, and the sterol is not found in other compartments (7). The cholesteryl ester associated with caveolae is reversible, that is, it can be effluxed to extracellular acceptors (7, 12). Once the caveolae are saturated with cholesteryl ester, the sterol begins to appear in an intracellular membrane compartment where it is irreversible, that is, it does not efflux to extracellular acceptors. The mechanism of how cholesteryl ester translocates from caveolae to internal membranes is not known.

In the present study we hypothesized that cholesteryl ester is transported from caveolae to internal membranes by a lipid-protein complex similar to the complex that transports newly synthesized cholesterol to caveolae. We demonstrate that caveolin directly binds to cholesteryl ester and that a HDL-de-
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Dependent and SR-BI-dependent intracellular lipid-protein complex is involved in the translocation of cholesteryl esters from caveolae to an intracellular membrane compartment. These data demonstrate 1) a mechanism for the intracellular trafficking of cholesteryl esters, 2) the regulation of caveolin-dependent sterol transport, and 3) the requirement of caveolin acylation in the directional transport of sterol.

EXPERIMENTAL PROCEDURES

Materials—RPMI medium 1640, Ham’s F-12 medium, Geneticin, calf serum, glutamine, trypsin-EDTA, LipofectAMINE, and penicillin/streptomycin were from Invitrogen. The Bradford assay kit was from Bio-Rad. Percoll, PVDF membrane, and Tween 20 were purchased from Sigma. OptiPrep was from Invitrogen. The anti-caveolin IgG (caveolin-1) and anti-annexin II IgG were from Transduction Laboratories. The anti-HSP56, anti-cytochrome C, and anti-cyclophilin A IgGs were from Affinity BioReagents. Horseradish peroxidase-conjugated IgGs were supplied by Cappel (West Chester, PA). Super Signal chemiluminescent substrate was purchased from Pierce. Caveolin cell lines (2, 4, 13) and SR-BI cell lines (7, 11) were all the same as described previously. [1,2,6,7-3H]cholesteryl oleate (93 Ci/mmol), [1,2,6,7-3H]cholesteryl-oleoyl ether (47 Ci/mmol), and 125I-Na (1 mCi/ml) were purchased from PerkinElmer Life Sciences. Isopropanol was purchased from Fisher, and hexane was from EM Science (Darmstadt, Germany).

Buffers—Buffer A consisted of 20 mM Tris, pH 7.6, 137 mM NaCl, 0.5% (v/v) Tween 20. Buffer B consisted of 25 mM MES, pH 6.5, 0.15 mM NaCl, 1% (v/v) Triton X-100, 60 mM octylglucoside, 0.1% (w/v) SDS. Buffer C (5 x sample buffer) was 0.31 M Tris, pH 6.8, 2.5% (w/v) SDS, 50% (v/v) glycerol, and 0.125% (w/v) bromphenol blue.

Cell Culture—Ida-7 (a LDL receptor-negative CHO cell line) cells were generously provided by Dr. Monty Krieger (Massachusetts Institute of Technology, Cambridge, MA). CHO lines (plus-minus, SR-BI) (7) were cultured in Ham’s F-12 medium containing bovine serum, 2 mM t-glutamine, 100 units/ml penicillin, and streptomycin. The cells were seeded (5 x 10^5 cells/plate) in 10-cm dishes on day 0 and used when 90% confluent (day 4). Medium was changed daily. The cells were then harvested at 4°C for 4 h at 4°C, the appropriate cell lysate (200 µg/ml) plus 30 mg/ml of bovine serum albumin in 25 mM MOPS, pH 6.5, 0.15 mM NaCl, 1% (v/v) Triton X-100, 60 mM octylglucoside, and 0.1% (w/v) SDS or RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0). Blocked beads were then used to preclear the experimental fractions that had been adjusted to 1% (v/v) Triton X-100, 60 mM octylglucoside, and 0.1% (w/v) SDS. Precleared fractions were then incubated for 18 h at 4°C with the appropriate antibody (2 µg of IgG/sample) before adding blocked protein A-Sepharose beads and incubating an additional 2 h at 4°C. The beads were collected by centrifugation and washed five times in high salt RIPA buffer (500 mM NaCl). Precipitated proteins were detected by immunoblots or silver stains. For the silver staining experiment 30 g/ml Geneticin. The cells were used on day 4.

Isolation and Labeling of Lipoproteins—LDL (d 1.05 g/ml) and HDL (d < 1.05 g/ml) were isolated by ultracentrifugation using the analysis of variance procedure of SigmaPlot. When appropriate, the fractions were then purified by ultracentrifugation.

RESULTS

Cytosolic Pool of Cholesteryl Ester—We previously used CHO cells to demonstrate that SR-BI-dependent selective cholesteryl ester uptake occurs in caveolae and that the sterol is subsequently translocated to an internal membrane compartment; however, we did not elucidate the transport mechanism (7, 8, 11). In addition, we previously described a novel lipid-protein complex that transports cholesterol from the endoplasmic reticulum through the cytosol to caveolae independent of vesicles (2, 4). We hypothesized that a similar lipid-protein complex may be involved in the translocation of cholesteryl esters from caveolae to an intracellular membrane compartment. If this hypothesis is correct than HDL-derived cholesteryl ester should first appear in a caveolae subcellular fraction, followed by the cytosol, and finally internal membranes. To test this, CHO cells expressing SR-BI (7, 11) were incubated with 10 µg/ml of [3H]cholesteryl ester or [3H]cholesteryl ether-labeled HDL for the indicated times. The cells were then washed and fractionated as described (2, 18). Fig. 1 demonstrates that radiolabeled cholesteryl ester (panel A) and ra-
diolabeled cholesterol ether-labeled HDL (Fig. 2B) associated with caveolin and caveolin-associated proteins were immunoisolated, and the precipitates were used for immunoblot analysis. Fig. 4 demonstrates that caveolin IgG also precipitated four bands from LPDS- and LDL-treated cells at 18, 22, 40, and 56 kDa. Caveolin IgG also precipitated four bands that were not detected in control experiments (means ± S.D.). The data are from four independent experiments.

Lipid-Protein Complex—We next determined whether the diolabeled cholesterol ether-labeled HDL was bound to caveolin and whether caveolin was associated with cholesterol ether. CHO cells expressing SR-BI (7) were incubated with [3H]cholesteryl ester or [3H]cholesterol ether-labeled HDL for 30 min, washed, and then processed to isolate cytosol. The cells were washed and subfractionated to isolate cytosol, and internal membranes were resolved by SDS-PAGE and immunoblotted for caveolin. In addition, an aliquot of each fraction was processed for immunoblot analysis. Fig. 5 demonstrates that the 18-, 22-, 40, and 56 kDa bands were detected in LPDS- and LDL-treated cells at 15 min. The data are from four independent experiments (means ± S.D.).

To confirm that the sterol that immunoprecipitated with caveolin was bona fide cholesteryl ester, we performed gas chromatography-mass spectrometry analysis on the caveolin immunoprecipitate. CHO cells expressing SR-BI were incubated with HDL for 30 min, washed, and then processed to isolate cytosol. The samples were then prepared as described for Fig. 2, and the immunoprecipitate was extracted with isopropanol-hexane and derivatized with N,O-bis(trimethylsilyl)acetamide. Fig. 3 demonstrates that the retention time of the GC column was identical for an authentic cholesteryl oleate standard (Panel A) and the lipid associated with caveolin (Panel C). Four distinctive peaks were generated in the mass spectra of the cholesteryl oleate standard (Panel B). Importantly, the same four peaks were generated from the lipid associated with immunoprecipitated caveolin, demonstrating that cholesterol oleate is bound to caveolin (Panel D).

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Fig. 1. Time course of cholesteryl ester uptake. CHO cells transfected with human SR-BI (7) were incubated with 10 μg/ml of [3H]cholesteryl ester or [3H]cholesterol ether-labeled HDL for the indicated times. The cells were washed and subfractionated to isolate cytosol, and internal membranes were resolved by SDS-PAGE and immunoblotted for caveolin. In contrast, the nonspecific IgG did not precipitate any proteins (2, 4). The cells were treated with 5% LPDS, 10 μg/ml HDL, or 10 μg/ml LDL for 30 min and then processed to isolate cytosol. Caveolin along with caveolin-associated proteins were immunoisolated from the cytosol with caveolin IgG, and the precipitated material was resolved by SDS-PAGE and silver-stained to visualize proteins. Fig. 4 demonstrates that four bands were detected in LPDS- and LDL-treated cells at 18, 22, 40, and 56 kDa. Caveolin IgG also precipitated four bands from cells treated with HDL; however, the 56-kDa band was replaced with a 36-kDa band. To identify the protein bands, the cells were treated as described in the legend to Fig. 4, caveolin and caveolin-associated proteins were immunoisolated, and the precipitates were used for immunoblot analysis. Fig. 5 demonstrates that the 18-, 22-, 40-, and 56-kDa bands that immunoisolated with caveolin IgG from LPDS- or LDL-treated cells were cyclophilin A, caveolin

cyclophilin 40, and HSP56, respectively. Cyclophilin A, caveolin, and cyclophilin 40 were also found in the cytosol of HDL-treated cells, but HSP56 no longer co-precipitated with caveolin. The 36-kDa band found in the cytosol of HDL-treated cells was identified as annexin II. Identical results were obtained when IgGs for the different proteins were used for the immunoprecipitations (data not shown).

To determine whether the HDL-dependent association of annexin II with caveolin was SR-BI-dependent, we added 10 \( \mu g/ml \) HDL to control CHO cells (not transfected with SR-BI) and cells expressing SR-BI for 30 min. The cytosol was isolated, and caveolin and caveolin-associated proteins were then immunoprecipitated with caveolin IgG or annexin II IgG as described above. The precipitated material was resolved by SDs-PAGE, and the proteins were identified by immunoblot analysis. Fig. 6 demonstrates that HDL did not induce the association of caveolin and annexin II in CHO cells not transfected with SR-BI. In contrast, when HDL was added to cells transfected with SR-BI (CHO-SR-BI) caveolin IgG precipitated both caveolin and annexin II, and annexin II IgG precipitated both annexin II and caveolin.

**Caveolin Palmitoylation**

Caveolin is acylated at three positions (Cys\(^{133} \), Cys\(^{143} \), and Cys\(^{156} \)). We previously demonstrated that acylation of positions 143 and 156, but not position 133, was necessary for the formation of the caveolin-cholesterol chaperone complex (4). To determine whether acylation of caveolin is necessary for the association of annexin II with caveolin, we used our established cell lines (L1210) that express wild-type caveolin, caveolin lacking acylation at either position 143 and 156, or caveolin lacking all three sites of acylation (4). L1210 cells do not endogenously express caveolin but do express SR-BI (4). These cells were treated with 10

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**Fig. 3.** Gas chromatography-mass spectrometry analysis of caveolin-associated lipid. CHO cells transfected with human SR-BI (7) were incubated with 10 \( \mu g/ml \) of HDL for 30 min at 37 \( ^{\circ} \)C. The cytosol was then isolated by centrifugation on a Percoll gradient followed by a 400,000 \( \times \) g centrifugation to remove any residue-contaminating membranes. Caveolin IgG (2 \( \mu g/ml \)) was added to aliquots of cytosol (200 \( \mu g \)). The IgGs were precipitated with protein A-Sepharose, and the precipitated material was extracted with isopropanol-hexane and derivatized with \( N,O \)-bis(trimethylsilyl) trifluoroacetamide. The sample was then analyzed by gas chromatography, and the lipid was identified by mass spectrometry. **A**, GC retention time with an authentic cholesteryl oleate standard. **B**, mass spectra of an authentic cholesteryl oleate standard. **C**, GC retention time of lipids associated with caveolin IgG immunoprecipitates. **D**, mass spectra of lipids associated with caveolin IgG immunoprecipitates. The data are representative of three independent experiments.
The selective uptake of HDL-derived cholesteryl ester is mediated by SR-BI; however, the mechanism(s) whereby cholesteryl ester is transferred from HDL to the plasma membrane and then subsequently transported to intracellular locations is not understood. In the present study we focused on determining the mechanism for the internalization of cholesteryl ester from the plasma membrane to internal membranes. Because of the limited solubility of cholesteryl esters in aqueous environments, it seems likely that trafficking would occur in a vesicle. However, selective uptake of cholesteryl esters occurs independently of HDL particle uptake and SR-BI internalization (10), suggesting that a classical endocytotic mechanism is not involved. In CHO cells caveolae are the initial sites of cholesteryl ester accumulation in the plasma membrane (7, 8, 11), which suggests a possible role of caveolae in cholesteryl ester internalization. We previously demonstrated that caveolin can form a lipid-protein chaperone complex and facilitate the direct interaction of HDL particles with caveolae (12, 13) and proposed that caveolin is required for the rapid internalization of cholesteryl esters from HDL (14). The selective uptake of HDL-derived cholesteryl ester is affected by the presence of HDL (15, 16), and the presence of HDL is required for the internalization of cholesteryl ester into caveolae (17). In addition, caveolin is required for the internalization of cholesteryl ester into caveolae (18). The selective uptake of HDL-derived cholesteryl ester is mediated by SR-BI; however, the mechanism(s) whereby cholesteryl ester is transferred from HDL to the plasma membrane and then subsequently transported to intracellular locations is not understood. In the present study we focused on determining the mechanism for the internalization of cholesteryl ester from the plasma membrane to internal membranes. Because of the limited solubility of cholesteryl esters in aqueous environments, it seems likely that trafficking would occur in a vesicle. However, selective uptake of cholesteryl esters occurs independently of HDL particle uptake and SR-BI internalization (10), suggesting that a classical endocytotic mechanism is not involved. In CHO cells caveolae are the initial sites of cholesteryl ester accumulation in the plasma membrane (7, 8, 11), which suggests a possible role of caveolae in cholesteryl ester internalization. We previously demonstrated that caveolin can form a lipid-protein chaperone complex and facilitate the direct interaction of HDL particles with caveolae (12, 13) and proposed that caveolin is required for the rapid internalization of cholesteryl esters from HDL (14). The selective uptake of HDL-derived cholesteryl ester is affected by the presence of HDL (15, 16), and the presence of HDL is required for the internalization of cholesteryl ester into caveolae (17). In addition, caveolin is required for the internalization of cholesteryl ester into caveolae (18).
transport of newly synthesized cholesterol from the endoplasmic reticulum to caveolae (2, 4). We hypothesize that the same or a similar lipid-protein complex may transport cholesteryl esters from caveolae to internal membranes. In the present study we have described a novel mechanism whereby caveolin binds to and translocates cholesteryl ester from caveolae to intracellular membranes.

We (2, 4, 13) and others (21) have demonstrated that caveolin can exist in the cytosol as part of a nonvesicle lipid-protein complex. The present study demonstrates that cholesteryl ester associates with caveolin in a protein complex consisting of cyclophilin 40, cyclophilin A, and annexin II. Annexin II has been associated with lipid rafts and sterol-dependent membrane cycling previously (22–24). These previous studies and the presence of a 36-kDa band in the silver stain gel lead us to investigate the possibility of the involvement of annexin II in caveolin-dependent cholesteryl ester trafficking. The exact site(s) of interaction between annexin II and caveolin is not known. Annexin II does not contain a classical caveolin-binding motif that mediates the interaction of numerous proteins with caveolin (1). It is possible that annexin II does not interact with caveolin directly but associates with caveolin by binding cholesteryl ester. The mechanism of interaction between these proteins and cholesteryl ester requires further study.

The ability of HDL (outside the cell) to alter the composition of the caveolin-containing cytosolic lipid-protein complex (inside the cell) suggests that HDL somehow generates a signal that modulates the composition of the complex. In addition, this signal is specific for HDL because lipoprotein-deficient serum and LDL did not alter the lipid-protein complex. Although we do not know what this putative signal consists of, we did demonstrate that the ability of HDL to alter the lipid-protein complex is SR-BI-dependent (Fig. 6). At least two mechanisms are possible. The binding of HDL to SR-BI may stimulate a signaling cascade that indirectly alters the lipid-protein complex. Alternatively, the uptake and association of cholesteryl ester with caveolin may somehow directly alter the composition of the lipid-protein complex. Although to date it has not been demonstrated that SR-BI is directly involved in any signaling mechanism, CD36, a similar class B scavenger receptor, has been shown to activate nonreceptor tyrosine kinases (25–27). The putative role of SR-BI in signaling will require additional study.

The acylation of caveolin residue 133 is required for the binding of cholesteryl ester (see “Experimental Procedures”). The present study demonstrates that two types of cholesteryl esters from caveolae to internal membranes. In the present study we have described a novel mechanism whereby caveolin binds to and translocates cholesteryl ester from caveolae to intracellular membranes.

FIG. 8. Acylation of caveolin residue 133 alters the regulation of cholesteryl ester internalization and PC internalization.

The present data demonstrate that caveolin associates with cholesteryl ester and annexin II in a lipid-protein complex that appears to be involved in the internalization of cholesteryl esters from caveolae to internal membranes. The present data and previously published data (2, 4) suggest that two types of caveolin-containing lipid-protein complexes can exist: one for transporting newly synthesized cholesterol from the endoplasmic reticulum to caveolae (efflux) and one for transporting cholesteryl esters from caveolae to internal membranes (uptake). In addition, the binding of HDL to SR-BI can promote the switch from a cholesterol-transporting complex to a cholesteryl ester-transporting complex, although the mechanism is not known. The cholesterol-transporting complex would most likely increase cellular sterol concentrations, whereas the cholesteryl ester-transporting complex would most likely decrease cellular sterol concentrations. It is important to emphasize that the association of annexin II (Fig. 7) or in the rapid transport of HDL-derived cholesteryl esters is SR-BI-dependent (Fig. 6). At least two mechanisms are possible. The binding of HDL to SR-BI may stimulate a signaling cascade that indirectly alters the lipid-protein complex. Alternatively, the uptake and association of cholesteryl ester with caveolin may somehow directly alter the composition of the lipid-protein complex. Although to date it has not been demonstrated that SR-BI is directly involved in any signaling mechanism, CD36, a similar class B scavenger receptor, has been shown to activate nonreceptor tyrosine kinases (25–27). The putative role of SR-BI in signaling will require additional study.

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