Stabilization of Caveolin-1 by Cellular Cholesterol and Scavenger Receptor Class B Type I†

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ABSTRACT: Caveolae are 50−100 nm plasma membrane invaginations, which function in cell signaling, in transcytosis, and in regulating cellular cholesterol homeostasis. These subcompartments of the plasma membrane are characterized by the presence of caveolin proteins. Recent studies have indicated that caveolae may be involved in the regulation of cellular cholesterol efflux to high-density lipoproteins (HDL), as well as selective cholesteryl ester uptake mediated by scavenger receptor class B type I (SR-BI). In the present studies, we show that caveolin-1 expression in HEK-293T cells has no effect on SR-BI-mediated cellular cholesterol efflux to reconstituted HDL. However, SR-BI-mediated selective cholesteryl ester uptake is significantly inhibited by caveolin-1. Interestingly, we also found that SR-BI, but not CD36, can induce the dramatic stabilization of the caveolin-1 protein, independently of its transcriptional control. On the other hand, caveolin-1 has little effect on SR-BI stability, but clearly increases CD36 stability. Since SR-BI expression has been shown to increase cellular cholesterol levels, we next examined the effect of cholesterol itself on caveolin-1 stabilization and localization. When cells were loaded with cholesterol, we observed the dramatic stabilization of caveolin-1 with significant clustering of caveolin-1 at the cell surface. In addition, a palmitoylation-deficient caveolin-1 mutant was still responsive to cholesterol-induced stabilization, indicating that palmitoylation of caveolin-1 is not required for the cholesterol-induced stabilization of caveolin-1. These results suggest an important role for cholesterol and SR-BI in the regulation of caveolin functioning, especially in cell types such as endothelial cells and macrophages, which can be dramatically affected by changes in their cholesterol content during the development of atherosclerosis.

Caveolae are 50−100 nm plasma membrane invaginations that participate in cell signaling (*1*), transcytosis (*2*, *3*), and regulating cellular cholesterol homeostasis (*4*). These subcompartments of the plasma membrane are characterized by the presence of caveolin proteins (*5*, *6*). Caveolins are expressed principally in terminally differentiated cell types such as fibroblasts, adipocytes, smooth and striated muscle cells, and endothelial cells (*7*).

The observation that caveolin-1 binds cholesterol specifically (*8*, *9*) suggests that caveolin-1 may play a direct role in the regulation of cellular cholesterol homeostasis. Additionally, caveolin-1 mRNA expression is under the positive control of cellular cholesterol levels, and SREBP-1 negatively regulates *CAV-1* gene transcriptional activity (*10*). This finding is also in agreement with our observations that treatment of NIH 3T3 with HDL_3[1] significantly decreases caveolin-1 expression levels (*11*). Early work has proposed that plasma membrane caveolae can mediate cellular cholesterol efflux to plasma (*12*). However, this property of caveolae may be cell-type-specific since we have found that reduced caveolin-1 levels in transformed NIH 3T3 cells result in increased cellular cholesterol efflux to HDL_3 (*11*).

The importance of HDL in cholesterol elimination has been suggested by several epidemiological studies that show an inverse correlation between the development of coronary artery disease and HDL−cholesterol levels (*13*−*17*). The formation of HDL and its residence in the circulation depend on ABCA1-mediated initial lipidation of apolipoprotein A-I

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[1] Abbreviations: ABCA1, ATP-binding cassette transporter A1; apoA-I, apolipoprotein A-I; CE, cholesteryl ester; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; HDL, high-density lipoprotein; SR-BI, scavenger receptor class B type I.

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(*18*, *19*). The HDL particles that are consequently formed also function as the acceptors of cellular cholesterol (*20*). Cholesterol ester from HDL (HDL−CE) may be transferred to hepatocytes or steroidogenic cells via the scavenger receptor class B type I (SR-BI), which mediates selective uptake of HDL−CE without HDL-particle uptake (*21*).

SR-BI is a transmembrane protein containing two predicted membrane-spanning domains, with one extracellular domain as well as a C-terminal and an N-terminal cytoplasmic tail (*22*, *23*). Interestingly, SR-BI is associated with caveolae (*22*). Selective HDL−CE uptake mediated by SR-BI has recently been shown to be mediated through caveolae (*24*). In agreement with this finding, Matveev et al. have shown that differentiation of THP-1 monocytes into macrophages is associated with caveolin-1 expression and increased SR-BI-mediated selective HDL−CE uptake (*25*). However, studies with caveolin-1-transfected J774 and RAW macrophages suggested that caveolin-1 might be a negative regulator of SR-BI-mediated selective HDL−CE uptake (*26*). In steroidogenic tissues, the relationship between caveolin-1 and SR-BI-mediated selective HDL−CE uptake does not appear to be clear (*27*). In rat ovarian granulosa cells, upon luteinization, SR-BI does not colocalize with caveolae, and increases in SR-BI expression and selective CE uptake are associated with a decline in caveolin-1 expression (*28*). However, in similar studies with mouse ovarian granulosa cells, luteinization was associated with a marked increase in SR-BI expression, but only a modest increase in SR-BI-mediated HDL−CE selective uptake. However, caveolin-1 expression in mouse granulosa cells was not affected by luteinization (*29*).

In previous studies, we have shown that caveolin-1 can inhibit selective HDL−CE uptake in mouse hepatocytes infected with adenovirus carrying the caveolin-1 cDNA (*30*). In the present study, we have further examined the role of caveolin-1 in the regulation of SR-BI function. In addition, we show that SR-BI-mediated cholesterol enrichment of the plasma membrane can dramatically increase the stability of caveolin-1. Our current results represent the first demonstration that cellular cholesterol levels directly affect the stability of the caveolin-1 protein, independently of transcriptional control.

EXPERIMENTAL PROCEDURES

Materials. Antibodies and their sources were as follows: anti-caveolin-1 IgG (mAb 2297; gift of Dr. Roberto Campos-Gonzalez, BD Transduction Laboratories) (*31*), anti-caveolin-2 IgG (mAb 65; gift of Dr. Roberto Campos-Gonzalez, BD Transduction Laboratories) (*32*), rabbit polyclonal anti-GFP, anti-cMyc, and anti-caveolin-1 IgG (N-20; Santa Cruz Biotechnology, Inc, Santa Cruz, CA), monoclonal antibody anti-FLAG (Sigma, St. Louis, MO). The cDNAs for caveolin-1, cMyc-SR-BI, V_5-CD36, and N-FLAG-CD36 were as previously described (*23*, *33*−*36*). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was obtained from Molecular Probes (Eugene, OR). [1α,2α-^{3}H]cholesterol was purchased from DuPont NEN (Boston, MA). All other reagents were analytical grade.

Cell Culture and Transfections. COS-7 and HEK-293T cells were grown in DMEM supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% FBS. Transfections were performed using the Effectene transfection reagent (QIAGEN, Chatsworth, CA), as per the manufacturer's instructions.

Protein Expression in Transfected Cells. The protein concentration was measured using the BCA protein assay (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as the protein standard. Equal amounts of protein for each sample were loaded and run on SDS−polyacrylamide 12% gels. After transfer to nitrocellulose, the expression levels of caveolin-1, SR-BI, CD36, and GFP were examined using specific antibodies.

Cellular Cholesterol Efflux. Human recombinant apoA-I was obtained as previously described (*37*). Reconstituted LpA-I complexes were prepared by the cholate dispersion/Bio-Beads removal technique (*38*) starting with a POPC/ApoA-I ratio of 80/1 (mol/mol). LpA-I complexes were reisolated by size-exclusion chromatography using a Superose 6 column. For efflux experiments, cells in complete medium were seeded in six-well plates at a density of 2×10^4 cells/well. After 24 h, the medium was replaced with DMEM supplemented with 5% FBS and 5 μCi/mL [^{3}H]-cholesterol dispersed in 0.1% ethanol (percent final volume of the medium) for 24 h. Twelve hours before the experiment, the medium was replaced with DMEM containing 0.2% BSA. Before each efflux experiment, the cells were washed three times with DMEM and then incubated with DMEM containing the reconstituted 50 μg/mL Lp2A-I and 0.2% BSA. Medium aliquots were taken at different times of incubation and treated as previously described (*39*). At the end of the experiment, the cells were solubilized in 0.5 N NaOH to determine the protein and [^{3}H]cholesterol content. The results presented are expressed as the percentage of labeled cholesterol remaining in the cells as a function of time.

DiI−HDL Uptake Experiments. For DiI uptake studies, DiI-labeled HDL particles were obtained from Intracel Corp. (Rockville, MD). Transfected cells were seeded in six-well plates in RPMI 1640 containing 10% FBS. DiI−HDL uptake experiments were performed as described by Acton and Rigotti (*40*). The cells were washed with medium alone and incubated with DiI−HDL (20 μg/mL in DMEM containing 0.2% fatty acid free BSA) for 5 h at 37 °C. The cells were subsequently washed with PBS, and DiI incorporation was determined after solubilization of the cells with DMSO. The fluorescence of the different extracts was then determined. In parallel wells, the protein content was determined by solubilizing the cells in 0.5 N NaOH.

Analysis of Cells by Immunofluorescence Microscopy. The primary antibody (rabbit polyclonal anti-Cav-1 IgG or anti-GFP IgG) was incubated for 1 h with the fixed cells in the presence of PBS containing 0.2% BSA and 0.1% Triton X-100. After three washes, the cells were incubated for 1 h with secondary antibody (Rhodamine red X-labeled goat $F(ab')_2$ anti-mouse IgG or FITC-labeled goat $F(ab')_2$ anti-rabbit IgG; Jackson Immunoresearch Laboratory). After the cells had been rinsed three times, the slides were mounted with Slow-Fade antifade reagent (Molecular Probes, Eugene, OR). The cells were observed using a BioRad Radiance 2000 laser scanning confocal microscope.

Cellular Cholesterol Loading Experiments. HEK-293T or COS-7 cells were first transfected with caveolin-1, GFP, or

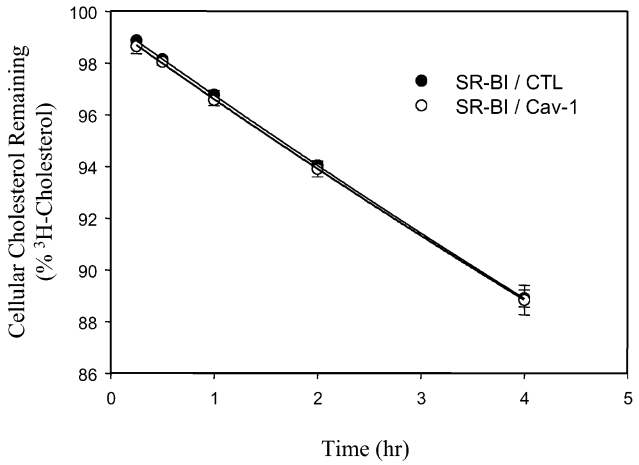

FIGURE 1: Caveolin-1 expression does not affect SR-BI-mediated cellular cholesterol efflux in HEK-293T cells. Transfected HEK-293T cells (SR-BI plus pCB7 (control vector) or SR-BI plus pCB7−Cav-1) were labeled in the presence of [^{3}H]cholesterol (see the Experimental Procedures) and incubated with Lp2A-I (50 μg/mL). Aliquots of the medium were removed at the indicated times and counted. Efflux is expressed as the percentage of [^{3}H]cholesterol remaining in the cells as a function of time (±standard deviation).

SR-BI cDNAs in a 10 cm dish. Twenty-four hours post-transfection, the cells were trypsinized and transferred in a six-well plate for 1 day. The medium was then replaced with varying concentrations of cholesterol (complexed to cyclodextrin; Sigma, St. Louis, MO). After a 24 h incubation, the cells were solubilized in lysis buffer. Equal amounts of protein for each sample were loaded and run on SDS−polyacrylamide 12% gels. After transfer to nitrocellulose, the expression levels of caveolins, SR-BI, and GFP were examined using specific antibodies.

RESULTS

Caveolin-1 Expression Does Not Affect SR-BI-Mediated Cellular Cholesterol Efflux. Previous studies have shown that caveolin-1 may have an important role in the regulation of cellular cholesterol efflux, but none of these reports have addressed the role of a possible interaction between SR-BI and caveolin-1 in this process. Therefore, we decided to determine the effect of caveolin-1 overexpression on SR-BI-mediated cellular cholesterol efflux. For this purpose, HEK-293T cells were transiently transfected either with SR-BI plus control vector (empty vector used to express the caveolin-1 cDNA) or SR-BI plus the caveolin-1 cDNA. The ability of reconstituted Lp2A-I to promote cellular cholesterol efflux from these cells was then examined.

The results presented in Figure 1 show that caveolin-1 overexpression in HEK-293T cells has no effect on SR-BI-mediated cholesterol efflux to Lp2A-I. It is important to note that transfection with SR-BI alone results in a significant increase in cellular cholesterol efflux as compared to that of mock-transfected cells (data not shown), as shown previously (*41*).

Caveolin-1 Expression Negatively Regulates SR-BI-Mediated Selective Cholesteryl Ester Uptake. We have previously shown that caveolin-1 can inhibit selective cholesteryl ester uptake in hepatocytes (*30*). To examine whether caveolin-1 could directly affect SR-BI-mediated selective HDL−CE uptake, HEK-293T cells were transfected either with SR-BI plus control vector or with SR-BI plus the caveolin-1 cDNA. The cells were incubated with HDL (using DiI as a fluorescent probe) for 5 h.

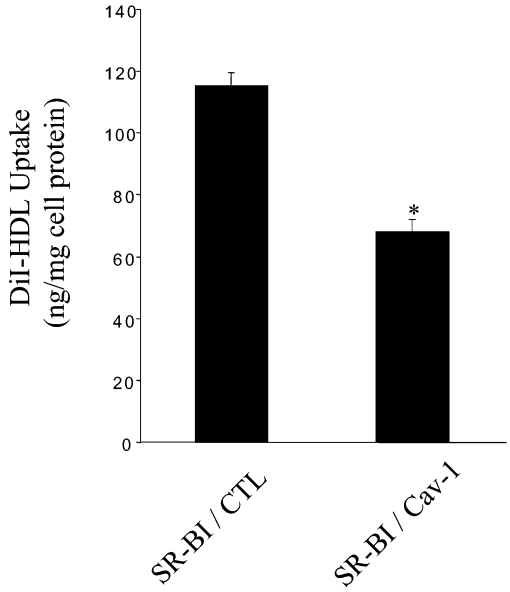

FIGURE 2: Caveolin-1 expression inhibits SR-BI-mediated selective HDL−CE uptake in HEK-293T cells. Transfected HEK-293T cells (SR-BI plus pCB7 (control vector) or SR-BI plus pCB7−Cav-1) were washed with PBS and incubated with DiI-labeled HDL for 5 h at 37 °C. The cells were subsequently washed with PBS, and DiI incorporation was determined after solubilization of the cells with DMSO. The fluorescence of the different extracts was then determined. In parallel wells, the protein content was determined by solubilizing the cells in 0.5 N NaOH. An asterisk indicates a significant difference, as compared with control cells ($p < 0.05$).

Figure 2 shows that in cells cotransfected with caveolin-1 and SR-BI, DiI uptake is significantly reduced by ∼30−40%, as compared to that in SR-BI-transfected cells. It is important to note that transfection with SR-BI alone results in a significant increase in selective cholesteryl ester uptake as compared to that of mock-transfected cells (data not shown), as observed previously (*42*).

Post-translational Stabilization of Caveolin-1 by Increasing Levels of SR-BI Expression. In the two experiments described above, we fortuitously observed that caveolin-1 protein levels in HEK-293T cells were increased by ∼8-fold when the cells were cotransfected with the SR-BI cDNA, as compared to cells transfected with caveolin-1 alone. Therefore, we next examined the expression of caveolin-1 as a function of SR-BI expression levels.

For this purpose, HEK-293T cells were transfected with a constant amount of the caveolin-1 cDNA and increasing amounts of the SR-BI cDNA (Figure 3A). In parallel, the same experiment was performed using the CD36 cDNA instead of the SR-BI cDNA (Figure 3B). CD36 is also a member of the class B scavenger receptors, but CD36 does not promote selective HDL−CE uptake.

The results presented in Figure 3 show that increasing SR-BI expression levels lead to a highly significant increase in caveolin-1 protein expression. In contrast, the expression of increasing amounts of CD36 has a smaller effect on caveolin-1 protein expression levels. It is important to note that both immunoblots performed for caveolin-1 detection were exposed for equivalent amounts of time. This result

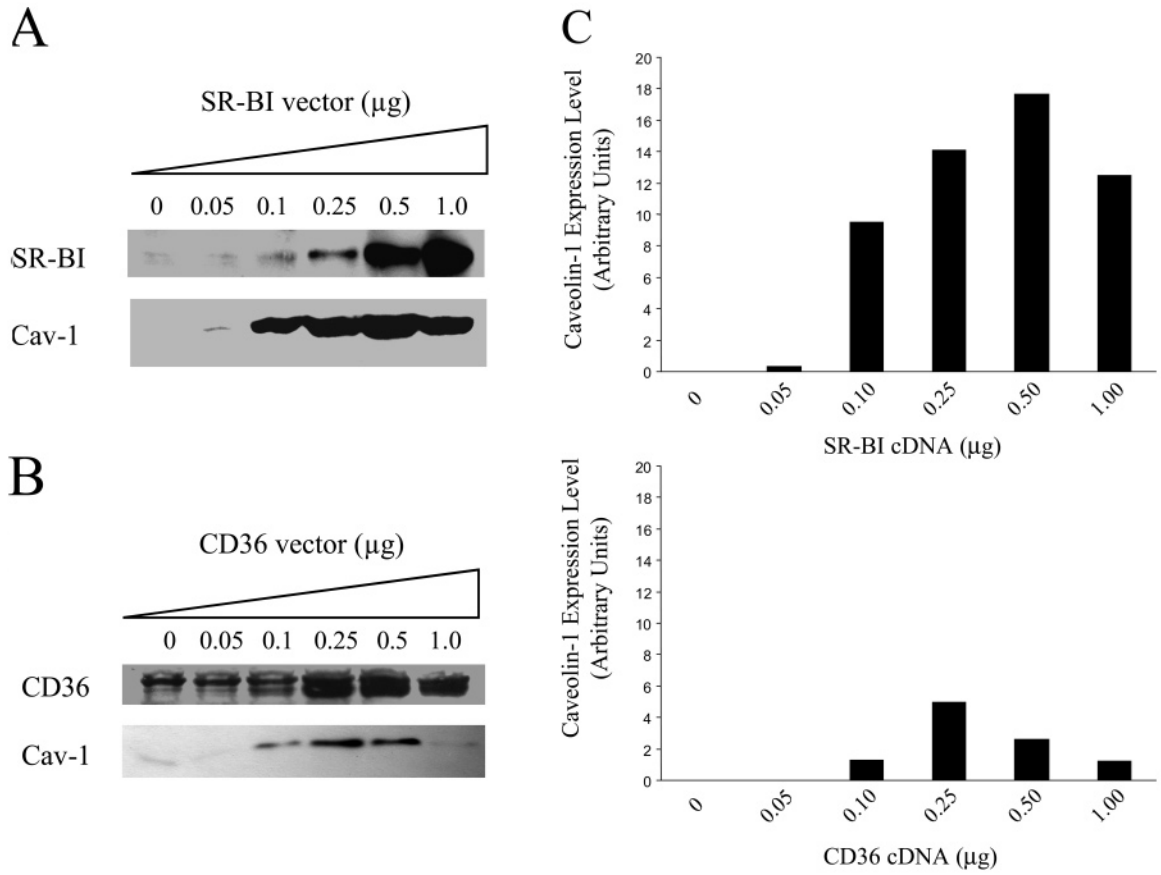

FIGURE 3: Caveolin-1 is post-translationally stabilized by SR-BI coexpression. HEK-293T cells were transfected with a constant amount of the caveolin-1 cDNA (1 μg) and increasing levels of the SR-BI (A) or CD36 (B) cDNAs (0−1 μg). Twenty-four hours post-transfection, the cells were solubilized and equivalent amounts of proteins were analyzed by SDS−PAGE. After transfer to nitrocellulose, expression levels for each protein were determined with specific antibodies. It is important to note that both immunoblots performed for caveolin-1 detection were exposed for comparable amounts of time. In panel C, densitometric analysis of these cotransfection experiments is presented. They indicate the fold increase in caveolin-1 protein levels in the presence of different amounts of SR-BI (A) or CD36 (B) cDNA. Note that caveolin-1 appears to be very efficiently stabilized by SR-BI (increased levels ~20×) but less efficiently by CD36 (~5×).

suggests that CD36 is not as efficient as SR-BI in stabilizing caveolin-1. We conclude that expression of SR-BI, and to much a lesser extent that of CD36, can dramatically stabilize the caveolin-1 protein product. As the caveolin-1 cDNA is expressed via a CMV-based vector, this stabilization effect is clearly independent of the normal transcriptional controls that regulate caveolin-1 gene expression.

Effect of Caveolin-1 Levels on SR-BI and CD36 Expression. To determine whether caveolin-1 could also affect the expression levels of SR-BI or CD36, we next transfected HEK-293T cells with constant amounts of the SR-BI or CD36 cDNAs and with increasing amounts of the caveolin-1 cDNA (Figure 4). In these experiments, we observed that increasing amounts of caveolin-1 expression only slightly increased SR-BI protein levels, except at the highest caveolin-1 cDNA levels used where SR-BI protein levels were actually reduced (Figure 4A). However, increasing caveolin-1 expression levels resulted in a dramatic increase in CD36 expression levels (Figure 4B). These results provide evidence that caveolin-1 may be an important regulator of CD36 stability.

Also, it is important to note that in separate cotransfection experiments, SR-BI had no effect on the expression of CD36, another known caveolar protein (data not shown).

Coexpression of SR-BI or CD36 with Caveolin-1 Induces Changes in Their Cellular Distribution. Confocal immunofluorescence microscopy was used to examine the effects of SR-BI and CD36 on the subcellular distribution of caveolin-1 in transfected COS-7 cells.

Figure 5A shows the localization patterns observed in cells singly transfected with Cav-1, SR-BI, or CD36. Note that Cav-1 is localized mainly at the plasma membrane, while SR-BI shows considerable intracellular staining. Furthermore, CD36 appeared to be retained intracellularly in a peri-nuclear Golgi-like compartment.

Next, we cotransfected COS-7 cells with either Cav-1 and SR-BI or Cav-1 and CD36. Interestingly, caveolin-1 had minimal effects on the cellular localization of SR-BI (Figure 5; compare panels A and B). However, while caveolin-1 remains associated with the plasma membrane, it is important to note that SR-BI colocalized with caveolin-1 (Figure 5B). Interestingly, CD36 was targeted to the plasma only when cotransfected with caveolin-1 (Figure 5; compare panels A and C). Thus, coexpression of CD36 with caveolin-1 shifts the cellular distribution of CD36 from the Golgi to the plasma membrane.

Increases in Cellular Cholesterol Levels Drive the Post-translational Stabilization of Caveolin-1. Previous studies have indicated that SR-BI has an important role in the regulation of cellular cholesterol homeostasis (*23, 43*). Moreover, Connelly et al. have shown that, under normal conditions, transfection of COS-7 cells with SR-BI, but not

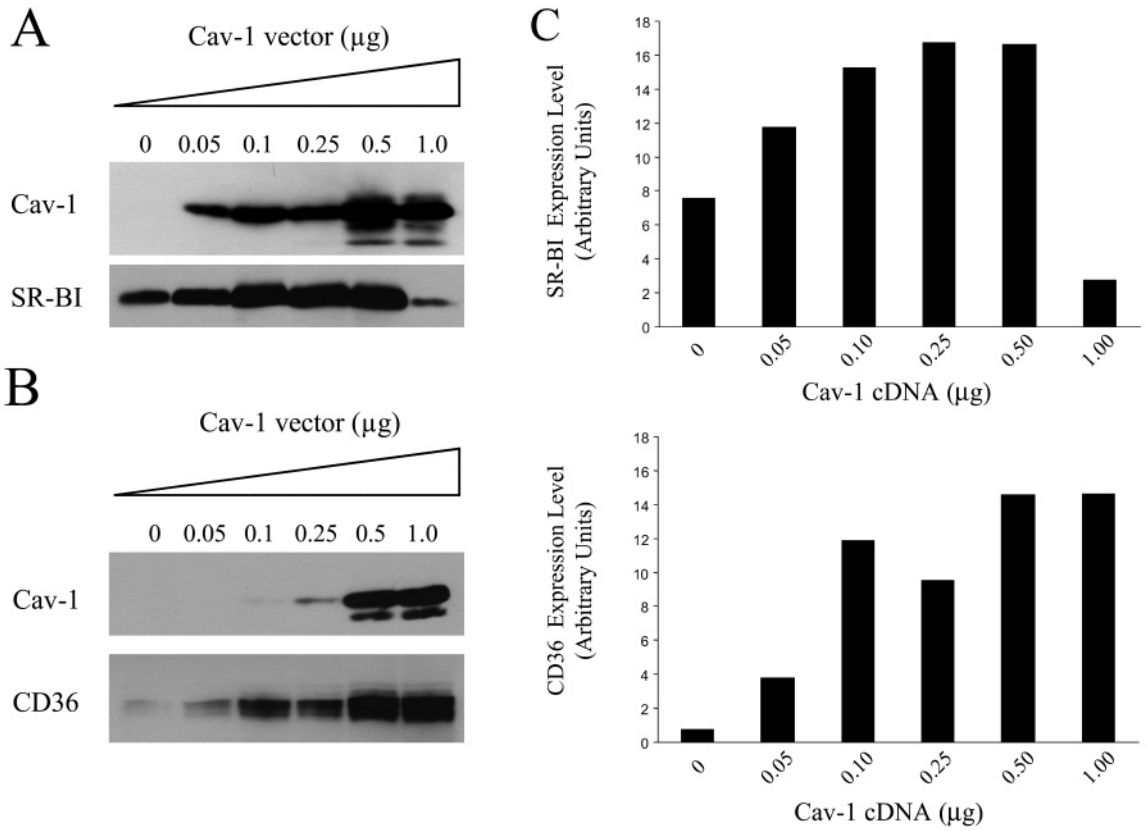

FIGURE 4: Caveolin-1 expression post-translationally stabilizes CD36, but not SR-BI. HEK-293T cells were transfected with a constant amount of the SR-BI or CD36 cDNAs (1 μg) and increasing levels of the caveolin-1 cDNA (0−1 μg). Twenty-four hours post-transfection, the cells were solubilized and equivalent amounts of proteins were analyzed by SDS−PAGE. After transfer to nitrocellulose, expression levels for each protein were determined with specific antibodies. It is important to note that both immunoblots performed for caveolin-1 detection were exposed for comparable amounts of time. In panel C, densitometric analysis of these cotransfection experiments is presented. They indicate the fold increase in SR-BI (A) or CD36 (B) protein in the presence of different amounts of the caveolin-1 cDNA. Note that CD36 is remarkably stabilized by caveolin-1 (increased levels ~10×) whereas SR-BI stabilization remains modest.

with CD36, leads to increased cellular cholesterol levels (*23*). Similarly, stable expression of SR-BI in human lung fibroblasts causes net increases in both cellular free cholesterol and phospholipid (*44*).

To determine whether the effect of SR-BI on caveolin-1 stabilization was related to a change in the cellular cholesterol levels or distribution, HEK-293T cells were transfected with caveolin-1 and subsequently loaded with cholesterol using cholesterol complexed with methyl-β-cyclodextrin (CD−Chol). Using varying concentrations of CD−Chol, we show that caveolin-1 protein levels increase dramatically (Figure 6A) with increased cellular cholesterol levels. As negative controls for this experiment, we also examined the effect of increasing cellular cholesterol concentrations on the expression levels of SR-BI and GFP. Importantly, increased cellular cholesterol has no effect on the expression levels of SR-BI (Figure 6B) or GFP (Figure 6C). Also, mock-transfected HEK-293T cells loaded with varying CD−Chol concentrations do not express any endogenous caveolin-1 (data not shown).

Increases in Cellular Cholesterol Levels Induce Changes in the Plasma Membrane Distribution of Caveolin-1. After cholesterol loading, confocal immunofluorescence microscopy was used to examine any possible changes in the localization of caveolin-1 in transfected COS-7 cells.

We observed that the pattern of caveolin-1 localization was dramatically affected by cellular cholesterol loading (Figure 7). In fact, we observed that caveolin-1 is now found in clusters at the surface of the plasma membrane in cells loaded with cholesterol. Some staining was also observed in a perinuclear region corresponding to the Golgi apparatus. In contrast, increasing cellular cholesterol levels had no effect on the localization of GFP in COS-7 cells (data not shown)

Palmitoylation Does Not Play a Role in the Stabilization of Caveolin-1 by Cholesterol. Previous studies have suggested a positive role for caveolin-1 palmitoylation in determining caveolin-1's ability to associate with and transport cholesterol. To examine the potential role of caveolin-1 palmitoylation in the stabilization of caveolin-1 by cholesterol, we next employed a well-characterized palmitoylation-deficient form of caveolin-1. In this caveolin-1 triple mutant, all three normally palmitoylated cysteine residues within caveolin-1 have been mutated to serine (C133, 143, 156S) (*35*).

HEK-293T cells were transfected with the cDNAs encoding wild-type caveolin-1 or the palmitoylation-deficient form of caveolin-1. Transfected cells were then incubated with 0 or 25 μg/mL CD−Chol. The results presented in Figure 8 directly show that palmitoylation of caveolin-1 is not required for its stabilization by cholesterol.

DISCUSSION

In the present study, we have shown that caveolin-1 regulates SR-BI-mediated selective HDL−CE uptake, but

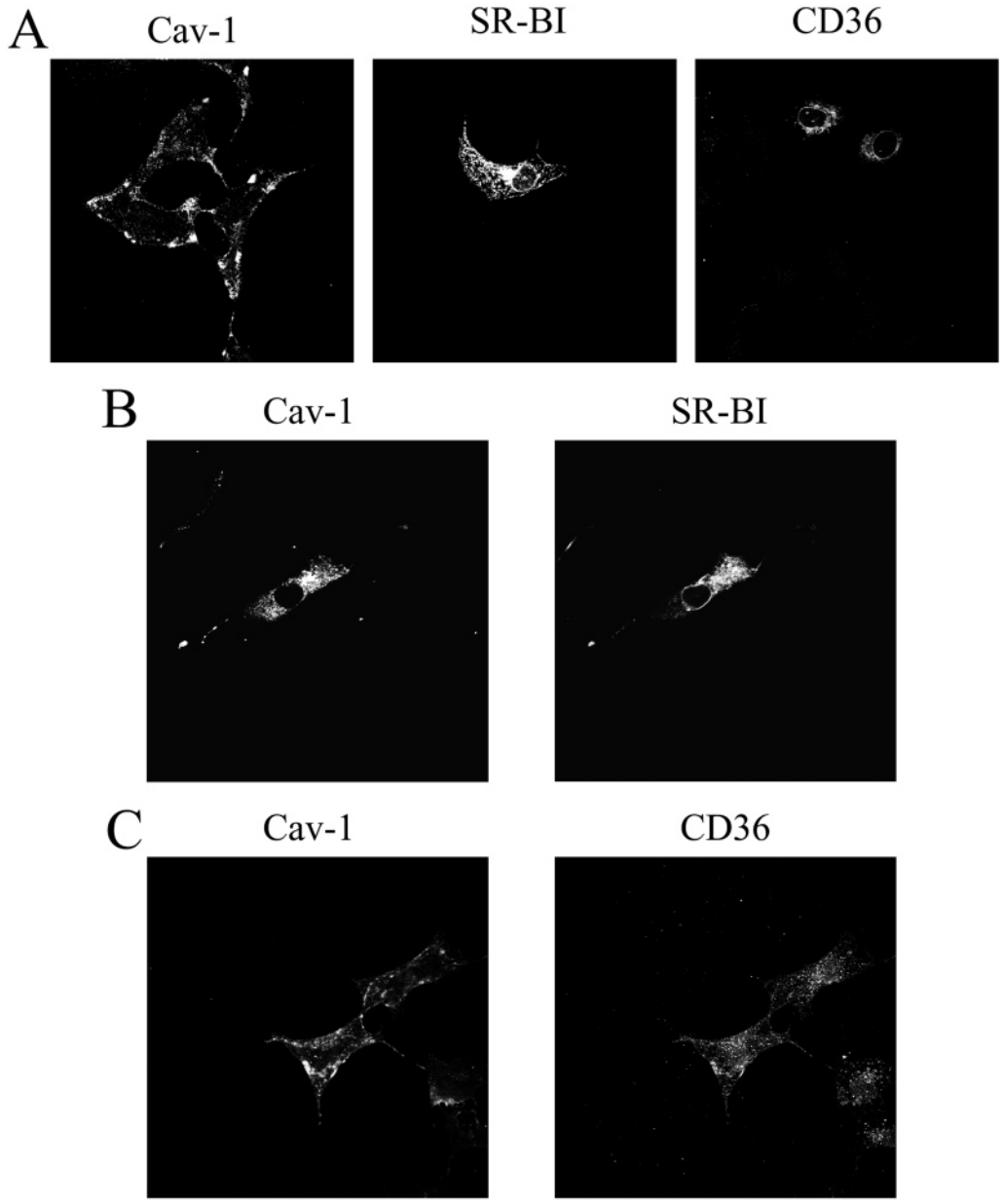

FIGURE 5: Immunolocalization of caveolin-1 in COS-7 cells coexpressing SR-BI or CD36. COS-7 cells were seeded into 12-well plates containing coverslips. The following day, the cells were transfected with the indicated construct(s). Twenty-four hours later, the cells were then immunostained and visualized by confocal fluorescence microscopy. (A) Cav-1, SR-BI, or CD36 alone. Panel A shows the distribution of Cav-1, SR−BI, and CD36 in singly transfected cells. Note that Cav-1 is localized mainly at the plasma membrane, while SR-BI shows considerable intracellular staining. Furthermore, CD36 appeared to be retained intracellularly in a peri-nuclear Golgi-like compartment. (B) Cav-1 and SR-BI. COS-7 cells were cotransfected with Cav-1 and SR-BI. Interestingly, caveolin-1 had minimal effects on the cellular localization of SR-BI (compare panels A and B). However, while caveolin-1 remains associated with the plasma membrane, it is important to note that SR-BI colocalized with caveolin-1. (C) Cav-1 and CD36. COS-7 cells were cotransfected with Cav-1 and CD36. Interestingly, CD36 was targeted to the plasma only when cotransfected with caveolin-1 (compare panels A and C). Thus, coexpression of CD36 with caveolin-1 shifts the cellular distribution of CD36 from the Golgi to the plasma membrane.

has no effect on SR-BI-mediated cholesterol efflux to HDL. More importantly, we found that SR-BI expression can also dramatically regulate the stability of caveolin-1. Mechanistically, we show that the SR-BI-mediated stabilization of caveolin-1 may be the result of an increase in the cellular cholesterol content or a change in its distribution. These results have broad implications for the regulation of SR-BI-mediated selective HDL−CE uptake and for the regulation of caveolin-1's role in repressing certain signal transduction pathways.

Caveolin-1 and Cellular Cholesterol Efflux. Previous studies have suggested a role for caveolin-1 in the regulation of cellular cholesterol efflux mediated by HDL. It was proposed that plasma membrane caveolae can mediate cellular cholesterol efflux to HDL or plasma (*12*). We previously reported that caveolin-1 down-regulation enhances cellular cholesterol efflux (*11*). However, we did not examine the effect of caveolin-1 on SR-BI in this process.

In this study, we show that caveolin-1 has no effect on the regulation of SR-BI-mediated cellular cholesterol efflux

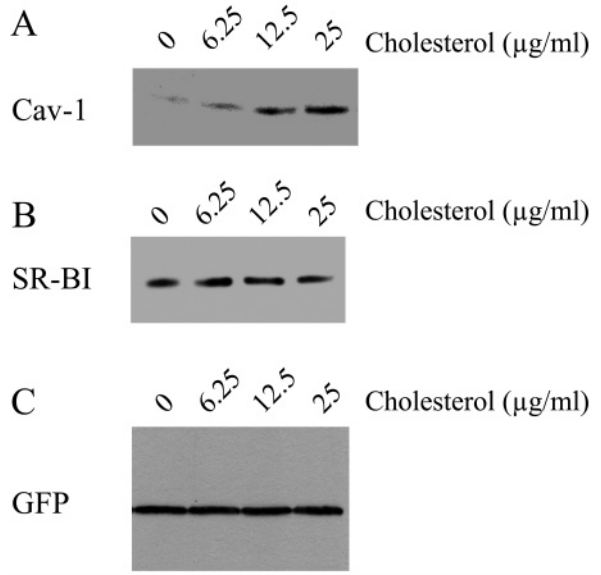

FIGURE 6: Cholesterol loading of HEK-293T cells increases caveolin-1 protein stability, but has no effect on the protein expression of GFP or SR-BI. HEK-293 T cells in 10 cm dishes were transfected with cDNAs encoding caveolin-1, GFP, or SR-BI. Twenty-four hours post-transfection, the cells were trypsinized and seeded into six-well plates. The following day, the cells were incubated with varying amounts of CD−Chol (cholesterol complexed with methyl β-cyclodextrin, Sigma). Cell extracts were then analyzed by SDS−PAGE/Western blotting ~24 h after cholesterol loading. Note that caveolin-1 protein levels increase dramatically with increased cellular cholesterol levels (A). However, increased cellular cholesterol has no effect on the expression levels of SR-BI (B) or GFP (C). Also, mock-transfected HEK-293T cells loaded with varying CD−Chol concentrations do not express any endogenous caveolin-1 (data not shown).

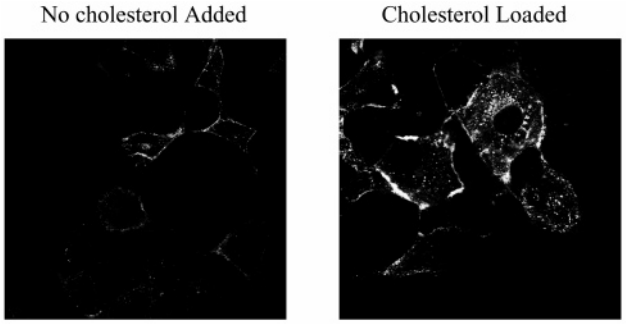

FIGURE 7: Immunofluorescence localization of caveolin-1 in COS-7 cells loaded with varying amounts of cholesterol. COS-7 cells in 10 cm dishes were transfected with the cDNA encoding caveolin-1. Twenty-four hours post-transfection, the cells were trypsinized and seeded into 12-well plates containing coverslips. The following day, the cells were incubated with 0 or 25 μg/mL CD−Chol. The cells were then immunostained with antibodies to caveolin-1 and visualized by confocal fluorescence microscopy. Note that the pattern of caveolin-1 localization is dramatically affected by cellular cholesterol loading. In fact, caveolin-1 is now found clustered at the surface of the plasma membrane in cholesterol-loaded cells. After cholesterol loading, Cav-1 immunostaining was also observed in a perinuclear region corresponding to the Golgi apparatus.

to Lp2A-I. This result is in agreement with previously published work from Ji et al. (*41*). These authors have correlated SR-BI expression in different cell lines with the ability of HDL to promote cellular cholesterol efflux from these cells. These cell lines include Fu5AH, a hepatocyte cell line, and J774, both of which do not express caveolin-1, and CHO and Y1-BS1, which express caveolin-1. The

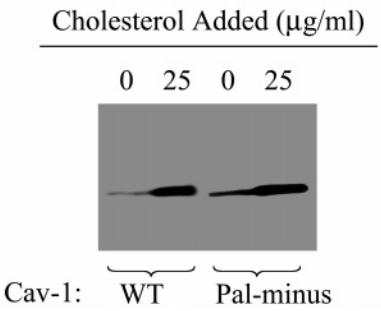

FIGURE 8: Palmitoylation does not play a role in the stabilization of caveolin-1 by cholesterol. HEK-293T cells were transfected with cDNAs encoding either wild-type caveolin-1 or a palmitoylation-deficient mutant of caveolin-1 (C133, 143, 156S). Twenty-four hours post-transfection, the cells were trypsinized and seeded into six-well plates. The following day, the cells were incubated with 0 or 25 μg/mL CD−Chol. The cell extracts were then analyzed by SDS−PAGE/Western blotting ~24 h after cholesterol loading.

ability of HDL or serum to promote cellular cholesterol efflux from these cells (CHO < J774 < Y1-BS1 < Fu5H) was not correlated with caveolin-1 expression, but only with SR-BI expression levels. These results suggest that other proteins have a more important role in the regulation of cellular cholesterol efflux to HDL. Furthermore, transient and stable expression of varying caveolin-1 levels were shown to increase cellular cholesterol synthesis and cycling, but had no effect on the initial rates of efflux to HDL (P. H. Links, A. G. McKenzie, X. Zha, P. G. Frank, Z. Yao, M. P. Lisanti, and Y. L. Marcel, submitted for publication).

We have also shown that caveolin-1 down-regulation in NIH 3T3 cells was associated with increased cellular cholesterol efflux to HDL_3 (*11*). This finding may suggest that the transformed cells used in our previous studies may have an altered regulation of cellular cholesterol homeostasis, as suggested by previous studies. For example, it has been demonstrated that tumors and transformed cells show diminished control of cholesterogenesis (see ref *45* and references therein). Most of these studies have in fact demonstrated enhanced cholesterol synthesis and, often, cholesterol enrichment of the plasma membrane. In addition, these transformed cells can also secrete lipid vesicles. Taken together, these properties may contribute toward enhanced cellular cholesterol efflux from cells in which caveolin-1 is down-regulated. Increased cholesterol synthesis in the transformed cell lines may be compensated by an increased cholesterol efflux—to maintain normal cellular cholesterol levels (*11*).

Caveolin-1 and the Regulation of SR-BI-mediated Selective HDL−CE Uptake. Overexpression of caveolin-1 in hepatocytes was shown to down-regulate selective HDL−CE uptake without changing SR-BI protein levels (*30*). However, we did not examine whether this was directly due to an effect of caveolin-1 on SR-BI. The present study suggests a direct effect of caveolin-1 on SR-BI-mediated selective HDL−CE uptake. Selective HDL−CE uptake mediated by SR-BI was recently shown to be mediated through caveolae in CHO cells (*24*). This group also showed that caveolin-1 expression might alter selective HDL−CE uptake in macrophages (*25*). However, these effects appeared to be dependent on the cell type used (*26*). In steroidogenic tissues, the relationship between caveolin-1 and SR-BI-mediated selective HDL−CE uptake is still unclear (*27*, *46*).

HDL−CE may be transferred, after binding of HDL to SR-BI, to what has been termed a reversible pool. This pool may be internalized within the cell or returned to HDL. The equilibrium may be affected by caveolae components, such as caveolin-1 and/or cholesterol. High levels of caveolin-1 protein and/or cholesterol may prevent the transfer of CE to intracellular stores. The mechanism for this inhibition may be due to an enhanced association of caveolin-1 with SR-BI, since SR-BI has been shown to coimmunoprecipitate with caveolin-1 (*22*, *47*). In that case, caveolin-1 may interact with one of the SR-BI intracellular domains, and this interaction may regulate SR-BI-mediated selective HDL−CE uptake.

This SR-BI/caveolin-1 interaction may be similar to the one proposed for p70 (*48*). p70 or CLAMP is a 70 kDa protein containing four PDZ domains. This protein has been shown to interact with the SR-BI C-terminal domain. Moreover, this SR-BI/p70 interaction may also regulate SR-BI expression and function (*48*). Therefore, caveolin-1 may act in a similar manner by directly inhibiting SR-BI-mediated selective HDL−CE uptake. An important role for the C-terminal domain has also been demonstrated in the case of SR-BII, an isoform of SR-BI containing an alternative C-terminal tail (*49*). SR-BII has been shown to have a reduced ability to promote selective HDL−CE uptake (*50*) due to an inhibitory effect of the alternate C-terminal tail (*23*).

Recent studies by Silver et al. have shown that when SR-BI is expressed in MDCK cells, HDL cholesterol and protein are selectively sorted after uptake (*51*). In these studies, SR-BI was primarily localized to the basolateral plasma membrane of the cells and HDL-derived cholesterol was secreted to the apical plasma membrane. Interestingly, MDCK cells express both caveolin-1 and caveolin-2. While caveolin-1 and caveolin-2 are associated together at the basolateral plasma membrane, caveolin-1 is found alone at the apical plasma membrane (*52*). Caveolin-2 may therefore not allow for an interaction between caveolin-1 and SR-BI and permit selective HDL−CE uptake. However, coexpression and colocalization of caveolin-1 and SR-BI may inhibit selective HDL−CE uptake.

Cholesterol and the Regulation of Caveolin-1 Protein Levels. Studies have shown that caveolin-1 expression is positively regulated by cellular cholesterol content at the level of transcriptional control (*10*, *53*). Cyclodextrin and HDL can both reduce endogenous caveolin-1 expression, while LDL can increase endogenous caveolin-1 expression levels (*11*, *53*). The role of cholesterol in the regulation of *CAV-1* transcriptional control has been evaluated by Fielding and co-workers (*4*, *23*). Their findings suggest that SREBP-1 can inhibit *CAV-1* transcription while activating other genes involved in the synthesis or uptake of cholesterol (*23*). These studies were performed examining endogenous caveolin-1 levels and suggest that increased cholesterol can lead to increased *CAV-1* transcriptional activity. However, it remained unknown whether caveolin-1 protein levels are post-translationally regulated by the cellular content of cholesterol.

Regulation of caveolin-1 expression at the protein level may also be important, especially in the case of changes in the plasma membrane cholesterol content. Furuchi et al. have shown that short-term treatment with methyl β-cyclodextrin is accompanied by the redistribution of caveolin-1 from plasmalemmal caveolae to other cellular organelles, such as the Golgi and the ER (*54*). Similar results have been obtained in the case of cells treated with cholesterol oxidase (*55*). Treatment with this enzyme leads to the internalization and redistribution of caveolin-1 to the ER and the Golgi apparatus. This treatment may therefore lead to the rapid degradation of caveolin-1.

It is therefore not surprising to observe that cholesterol loading of COS-7 or HEK-293T cells induces the dramatic stabilization of the caveolin-1 protein since plasma membrane cholesterol should be highly increased by cholesterol loading. In the case of SR-BI cotransfection experiments, caveolin-1 stabilization was even more pronounced. This effect may be due in part to the specific localization of SR-BI in caveolae (*22*) and the activity of SR-BI to alter the content and distribution of free cholesterol in plasma membrane microdomains. Transient or stable expression of SR-BI increases the plasma membrane pool of free cholesterol that is sensitive to oxidation by exogenous cholesterol oxidase (*23*, *43*, *44*, *56*).

Furthermore, SR-BI enlarges the fast kinetic pool of membrane free cholesterol that effluxes to cyclodextrin acceptors (*43*). SR-BI has also been shown to mediate HDL-dependent activation of endothelial nitric oxide synthase, an enzyme localized to caveolae (*57*). These findings indicate that SR-BI alters the free cholesterol content of membrane microdomains. The SR-BI-mediated stabilization of caveolin may occur in a similar manner due to an altered content or organization of caveolar cholesterol.

A role for caveolin-1 palmitoylation in cholesterol metabolism has been suggested by Uittenbogaard et al. (*58*). This study suggested that caveolin-1 palmitoylation is required for cholesterol binding of caveolin-1 and cholesterol transport to plasma membrane caveolae. However, we show here that cholesterol loading of HEK-293T cells also dramatically stabilizes a palmitoylation-deficient mutant of caveolin-1 at levels that are virtually identical to those obtained with wild-type caveolin-1. This result indicates that cholesterol binding to caveolin-1 does not necessarily require palmitoylation. This result is in direct agreement with our previous studies using bacterially expressed caveolin-1 (*59*). We have shown that recombinant bacterially expressed caveolin-1, which lacks palmitoylation, incorporates into reconstituted membrane vesicles in a cholesterol-dependent fashion. A cholesterol liposome content of 50% increases the incorporation of caveolin-1 by ~25-fold. Similar results were obtained by Murata et al. (*8*). In addition, Dietzen et al. (*35*) and Uittenbogaard et al. (*58*) have shown that palmitoylation of caveolin-1 is not necessary for its localization to plasmalemmal caveolae (*35*). Therefore, palmitoylation of caveolin-1 is not necessary for cholesterol binding and caveolae formation, but may increase the stability of caveolin oligomers (*60*). However, caveolin-1 palmitoylation may still affect the caveolin-1 affinity for cholesterol and the rate of the biosynthetic transfer of cholesterol to caveolae.

Regulation of SR-BI and CD36 Expression by Caveolin-1. Our results also suggest that caveolin-1 can positively regulate the stability of the CD36 protein, but caveolin-1 expression does not appreciably affect SR-BI expression (Figures 3 and 4). In addition, we show that caveolin-1 can induce the targeting of CD36 to the plasma membrane. Interestingly, Devaraj et al. showed that differentiation of human monocytes into macrophages is associated with

increased CD36 protein levels (*61*). This finding is therefore consistent with the results presented in the current study since differentiation of human THP-1 monocytes into macrophages is associated with increased caveolin-1 protein expression (*25*). The up-regulation/stabilization of CD36 by caveolin-1 suggests that caveolin-1 may play an important role in CD36 functioning and regulation. Additional studies are required to characterize how caveolin-1 contributes to the regulation of CD36 stability and function. In further support of these findings, CD36 has been shown to localize within plasma membrane caveolae (*62*).

Functional Significance. The role of cholesterol in the regulation of caveolin-1 stability may be important for several of its functions, especially relating to the pathogenesis of atherosclerosis. For example, we have shown that a decrease in caveolin-1 protein levels in endothelial cells can reduce the uptake of oxidized LDL uptake by these cells (*11*).

Another important function of caveolin-1 is the inhibition of endothelial nitric oxide synthase (eNOS) activity (*63*). SR-BI has also been shown to mediate HDL-dependent activation of endothelial nitric oxide synthase, an enzyme localized to caveolae (*57*). Therefore, hypercholesterolemia and an increased plasma cholesterol content in endothelial cells are likely to have negative effects on endothelium-derived relaxation. However, this effect may be counteracted by HDL (*11*, *47*).

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