THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 22, pp. 14881–14890, May 29, 2009

Regulation of Intracellular Cholesterol Distribution by Na/K-ATPase*

Received for publication, November 19, 2008, and in revised form, April 3, 2009 Published, JBC Papers in Press, April 10, 2009, DOI 10.1074/jbc.M109.003574

Yiliang Chen[‡], Ting Cai[‡], Haojie Wang[‡], Zhichuan Li[‡], Elizabeth Loreaux[§], Jerry B. Lingrel[§], and Zijian Xie[‡1]

From the [‡]Department of Physiology and Pharmacology, College of Medicine, University of Toledo, Toledo, Ohio 43614-2598 and the [§]Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45267

Recent studies have ascribed many non-pumping functions to the Na/K-ATPase. We show here that graded knockdown of cellular Na/K-ATPase α1 subunit produces a parallel decrease in both caveolin-1 and cholesterol in light fractions of LLC-PK1 cell lysates. This observation is further substantiated by imaging analyses, showing redistribution of cholesterol from the plasma membrane to intracellular compartments in the knockdown cells. Moreover, this regulation is confirmed in $\alpha1^{+/-}$ mouse liver. Functionally, the knockdown-induced redistribution appears to affect the cholesterol sensing in the endoplasmic reticulum, because it activates the sterol regulatory element-binding protein pathway and increases expression of hydroxymethylglutaryl-CoA reductase and low density lipoprotein receptor in the liver. Consistently, we detect a modest increase in hepatic cholesterol as well as a reduction in the plasma cholesterol. Mechanistically, $\alpha1^{+/-}$ livers show increases in cellular Src and ERK activity and redistribution of caveolin-1. Although activation of Src is not required in Na/K-ATPase-mediated regulation of cholesterol distribution, the interaction between the Na/K-ATPase and caveolin-1 is important for this regulation. Taken together, our new findings demonstrate a novel function of the Na/K-ATPase in control of the plasma membrane cholesterol distribution. Moreover, the data also suggest that the plasma membrane Na/K-ATPase-caveolin-1 interaction may represent an important sensing mechanism by which the cells regulate the sterol regulatory element-binding protein pathway.

The Na/K-ATPase, also called the sodium pump, is an ion transporter that mediates active transport of Na^+ and K^+ across the plasma membrane by hydrolyzing ATP (1, 2). The functional sodium pump is mainly composed of α and β subunits. The α subunit is the catalytic component of the holoenzyme; it contains both the nucleotide and the cation binding sites (3). So far, four isoforms of α subunit have been discovered, and each one shows a distinct tissue distribution pattern (4, 5). Interestingly, studies during the past few years have uncovered many non-pumping functions of Na/K-ATPase (6–10). Recently, we have demonstrated that more than half of the Na/K-ATPase may actually perform cellular functions other than ion pumping at least in LLC-PK1 cells (11). Moreover, the non-pumping pool of Na/K-ATPase mainly resides in caveolae and interacts with a variety of proteins such as Src, inositol 1,4,5-trisphosphate receptor, and caveolin-1 (12–14). While the interaction between Na/K-ATPase and inositol 1,4,5-trisphosphate receptor facilitates Ca^{2+} signaling (13) the dynamic association between Na/K-ATPase and Src appears to be an essential step for ouabain to stimulate cellular kinases (15). More recently, we report that the interaction between the Na/K-ATPase and caveolin-1 plays an important role for the membrane trafficking of caveolin-1. Knockdown of the Na/K-ATPase leads to altered subcellular distribution of caveolin-1 and increases the mobility of caveolin-1-containing vesicles (16).

Caveolin is a protein marker for caveolae (17). Caveolae are flask-shaped vesicular invaginations of plasma membrane and are enriched in cholesterol, glycosphingolipids, and sphingomyelin (18). There are three genes and six isoforms of caveolin. Caveolin-1 is a 22-kDa protein and is expressed in many types of cells, including epithelial and endothelial cells. In addition to their role in biogenesis of caveolae (19), accumulating evidence has implicated caveolin proteins in cellular cholesterol homeostasis (20). For instance, caveolin-1 directly binds to cholesterol in a 1:1 ratio (21). It was also found to be an integral member of the intracellular cholesterol trafficking machinery between internal membranes and plasma membrane (22, 23). The expression of caveolin-1 appears to be under control of SREBPs,[2] the master regulators of intracellular cholesterol level (24). Furthermore, knockout of caveolin-1 significantly affected cholesterol metabolism in mouse embryonic fibroblasts and mouse peritoneal macrophages (25). Because we found that the Na/K-ATPase regulates cellular distribution of caveolin-1, we propose that it may also affect intracellular cholesterol distribution and metabolism. To test our hypothesis, we have investigated whether sodium pump α1 knockdown affects cholesterol distribution and metabolism both *in vitro* and *in vivo*. Our results indicate that sodium pump α1 expression level plays a role in the proper distribution of intracellular cholesterol. Down-regulation of sodium pump α1 not only redistributes

* This work was supported, in whole or in part, by National Institutes of Health Grants HL-36573, HL-67963, and GM 78565.

[1] To whom correspondence should be addressed: Dept. of Physiology and Pharmacology University of Toledo, College of Medicine, 3000 Arlington Ave., Toledo, OH 43614-2598. Tel.: 419-383-4182; Fax: 419-383-2871; E-mail: zi-jian.xie@utoledo.edu.

[2] The abbreviations used are: SREBP, sterol regulatory element-binding protein; ER, endoplasmic reticulum; KO, knockout; LDL, low density lipoprotein; mCBM, caveolin-binding motif mutant; Mes, 4-morpholineethanesulfonic acid; MBS, Mes-buffered saline; NPC1, Niemann-Pick type C1; SCAP, SREBP cleavage-activating protein; YFP, yellow fluorescence protein; HMG, hydroxymethylglutaryl; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine.

TABLE 1
Sodium pump expression level in different LLC-PK1 derived cell lines

Cell line	Na^+/K^+-ATPase α1 subunit
	Content (%)
P-11	100 ← negative control cell
A4–11	40 ← knockdown cell
PY-17	10 ← knockdown cell
AAC-19	100 ← rescued cell

cholesterol between the plasma membrane and cytosolic compartments, but also alters cholesterol metabolism in mice.

EXPERIMENTAL PROCEDURES

Material—The antibodies and their sources are as follows: The monoclonal anti-α1 antibody (α6F) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. The polyclonal anti-Tyr(P)^{418}-Src antibody was from Biosource (Camarillo, CA). The polyclonal anti-SREBP2 antibody was from Cayman Chemical (Ann Arbor, MI). The polyclonal anti-HMG-CoA reductase antibody was from Upstate Biotechnology Inc. (Lake Placid, NY). The monoclonal anti-LDL receptor was from Calbiochem. All the other antibodies included in this study were from Santa Cruz Biotechnology (Santa Cruz, CA). Optitran nitrocellulose membrane was from Schleicher & Schuell. Enhanced chemiluminescence SuperSignal kit was purchased from Pierce. Lipofectamine 2000 was purchased from Invitrogen. An Amplex Red Cholesterol Assay Kit was purchased from Molecular Probes, Inc. (Eugene, OR). The Src inhibitor, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)-pyrazolo[3,4-*d*]pyrimidine (PP2), was from Calbiochem.

Cell Culture—The control LLC-PK1 and P-11 cell lines, caveolin-1 knockdown C2–9 cell line, the Na/K-ATPase α1 knockdown cell lines (A4–11, PY-17, and TCN23-19), the rat α1-rescued PY-17 cell line (AAC-19) or the caveolin-binding motif mutant rat α1-rescued PY-17 cell line (mCBM) and the α1 N terminus-YFP expressing LLC-PK1 cells (NT-YFP) were derived from the LLC-PK1 cells as previously described (14, 16, 26, 27). The relative level of Na/K-ATPase α1 in control, α1 knockdown, and rescued cell lines is listed in Table 1. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin (100 units/ml)/streptomycin (100 μg/ml), and 1 μg/ml puromycin in a 5% CO_2-humidified incubator. After cells reached 90% confluence, they were serum-starved for 12 h and used for experiments unless indicated otherwise.

Plasmid Constructs and Transfection—The rat α1 pump-null mutant (D371N) was generated as previously described (16). When the Na/K-ATPase α1 knockdown cells reached ~70% confluence, they were transfected with the plasmid or an empty vector by Lipofectamine 2000 as described previously (16). Experiments were performed 6 h after transfection.

Experimental Animal—Na/K-ATPase $\alpha1^{+/+}$ and $\alpha1^{+/-}$ mice were generated as previously described (3). Genomic DNA was obtained from tail biopsies and used for PCR-based genotyping. Adult mice at 16~20 weeks were used for the study. All mice were kept in a 12-h dark/light cycle and fed standard chow *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Toledo, Health Science Campus.

Phenotypic Analysis—Following a 4-h fast, mice were euthanized with sodium pentobarbital (150 mg/kg). The animal was weighed before the abdominal cavity was opened, and whole liver organ was carefully dissected and weighed. The live weight/body weight ratio was calculated. Subsequently, liver tissues were immediately frozen in liquid nitrogen and stored at −80 °C for cholesterol assay and Western blot analysis. Meanwhile, blood was drawn from the inferior vena cava, stored on ice for 20~30 min, and centrifuged at 2800 × *g* for 10 min. The supernatant (plasma) was transferred to a fresh Eppendorf tube and sent to the University Medical Center immediately for plasma lipid profile analysis.

Purification of Caveolin-rich Membrane Fractions—Caveolin-rich membrane fractions were obtained via sucrose gradient fractionation as described previously (14). Briefly, cells were washed with ice-cold phosphate-buffered saline and scraped in 2 ml of 500 mM sodium carbonate, pH 11.0. The cell lysates were homogenized by a Polytron tissue grinder (three 6-s bursts) and subjected to sonication (three 40-s bursts). The homogenates were then adjusted to 45% sucrose by addition of 2 ml of 90% sucrose in MBS (25 mM Mes, 0.15 M NaCl, pH 6.5) and placed at the bottom of ultracentrifuge tubes. The ultracentrifuge tubes were then loaded with 4 ml of 35% sucrose and 4 ml of 5% sucrose (both in MBS containing 250 mM sodium carbonate) and centrifuged at 39,000 rpm for 16–20 h in an SW41 rotor (Beckman Instruments). Twelve gradient fractions of 1 ml were collected from the top to the bottom. Among the fractions, fraction 4 and 5 were combined and diluted with 4 ml of MBS, then centrifuged at 40,000 rpm in a Beckman type 65 rotor for 1 h. The pellet was resuspended in 250 μl of MBS and is considered as caveolin-enriched caveolar fraction.

Separation of Cytosol and Cellular Membrane—Cells were washed with ice-cold phosphate-buffered saline and scraped in 2 ml of sucrose buffer A (30 mM histidine, 250 mM sucrose, 1 mM EDTA-Na, pH 7.4). The cell lysates were homogenized by a Polytron tissue grinder (three 6-s bursts) and subjected to sonication (three 40-s bursts). Then cell lysates were transferred to a 15-ml Falcon centrifuge tube and subjected to centrifugation at 1,000 rpm for 5 min to remove cell debris. The supernatants were transferred to ultracentrifuge tubes and were centrifuged at 100,000 × *g* in a Beckman type 65 rotor for 1 h. The supernatants (taken as cytosol fraction), were collected in the Eppendorf tubes. The pellets, taken as the membrane fraction, were resuspended in 250 μl of buffer A.

Western Blot Analysis and Immunostaining of Caveolin-1—Protein concentration of cell lysates was measured by Protein Assay Kit from Bio-Rad (Hercules, CA). Cell lysates with equal amounts of proteins were loaded onto the gel and separated on 10% SDS-PAGE, transferred to an Optitran membrane, and probed with corresponding antibodies. Protein signals were detected with an ECL kit and quantified using a Bio-Rad GS-670 imaging densitometer. Immunostaining of caveolin-1 was performed as previously described (16).

Cholesterol Assay—Cholesterol was measured by Amplex Red Cholesterol Assay Kit according to the manufacturer's instructions, and the signals were detected by a microplate spectrofluorometer (excitation: 560 nm; cut off: 570 nm; emission: 590 nm).

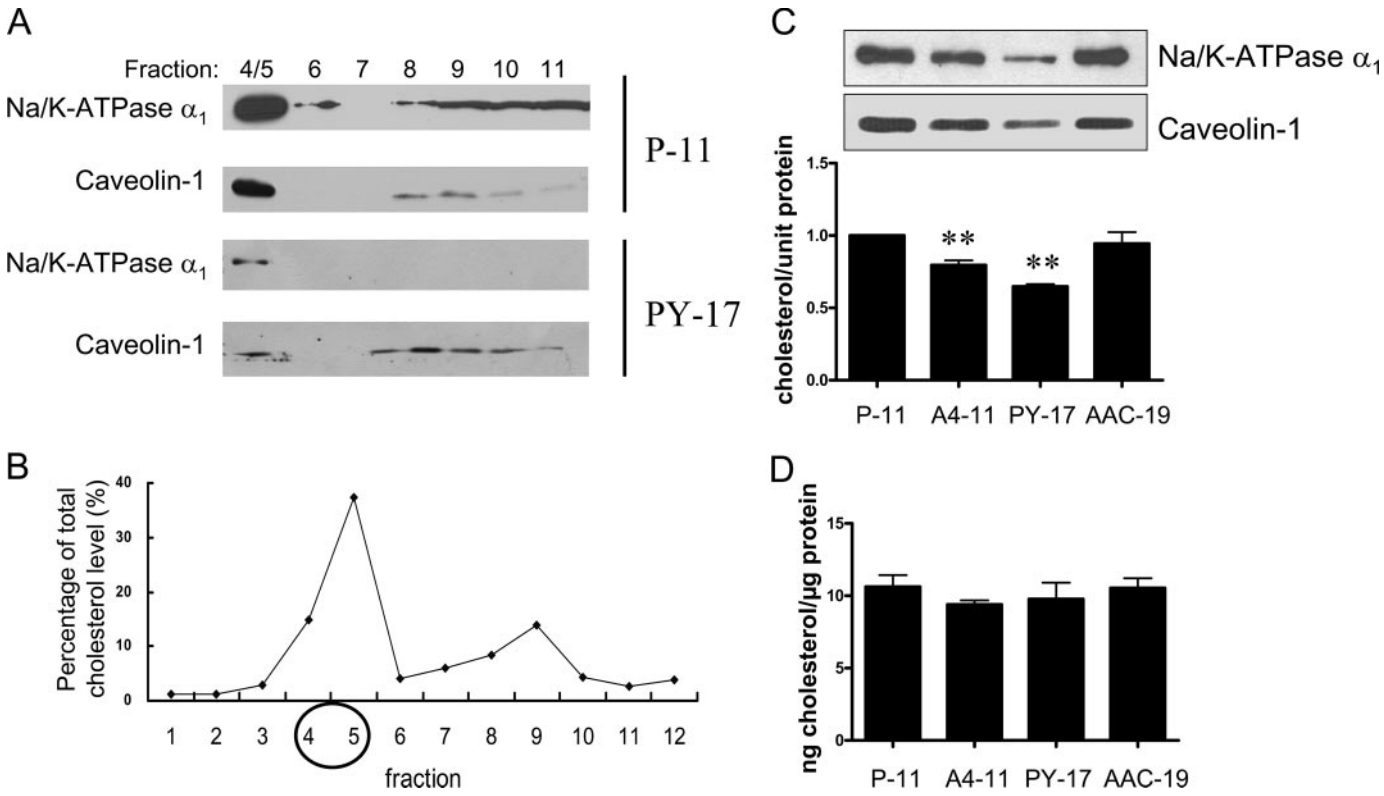

FIGURE 1. **Down-regulation of Na/K-ATPase α1 reduces both caveolin-1 and cholesterol in caveolar fractions.** *A*, cell lysates from P-11 and PY-17 cells were subjected to sucrose gradient fractionation as described under "Experimental Procedures." Caveolin-enriched fractions (4/5) together with fractions 6~11 were taken for Western blot analysis of Na/K-ATPase α1 and caveolin-1. A representative Western blot of five independent experiments is shown. *B*, cell lysates from P-11 cells were fractionated as in *panel A*, and all 12 fractions were assayed for cholesterol. The percentage of the cholesterol amount in each fraction was calculated. *C*, a representative Western blot of three experiments showing the amount of Na/K-ATPase α1 and caveolin-1 in caveolin-enriched fractions (4/5). Cholesterol content was measured from caveolin-enriched fractions. **, $p < 0.01$ compared with P-11 control cells, $n = 5$. *D*, cholesterol content was measured from total cell lysates, no difference was detected, $n = 3$.

Filipin Staining—Filipin staining was performed as described before (28). Filipin signals were viewed by a fluorescence microscope with a UV filter.

Data Analysis—Data are given as mean ± S.E. Statistical analysis was performed using the Student's *t* test and significance was accepted at $p < 0.05$.

RESULTS

Knockdown of the Na/K-ATPase α1 Redistributes Both Caveolin-1 and Cholesterol—We previously reported that the Na/K-ATPase α1 was concentrated in caveolae and interacted dynamically with caveolin-1 in LLC-PK1 cells (14). Moreover, knockdown of the α1 increased the mobility of caveolin-1, resulting in decreases in the number of caveolae and the amount of caveolin-1 in the plasma membrane (16). Because cholesterol plays an important role in regulation of the mobility of caveolin-1 and the formation of caveolae, we reasoned that knockdown of the α1 might also alter the membrane distribution of cholesterol. To test this hypothesis, cell lysates were fractionated using a detergent-free and carbonate-based density gradient fractionation procedure (14, 29). The low density fractions 4/5 prepared from the control LLC-PK1 cells contained ~50% of caveolin-1, and the Na/K-ATPase α1 and was taken as caveolin-1-enriched caveolar fraction (Fig. 1*A*) (16). Consistent with what was reported in the literature (30), this caveolar fraction also contained >50% of total cellular cholesterol (Fig. 1*B*). When caveolin-1, Na/K-ATPase α1, and cholesterol content were measured in this fraction prepared from different cell lines, we found that the Na/K-ATPase knockdown caused an α1 amount-dependent decrease in both caveolin-1 and cholesterol (Fig. 1*C*). Although cholesterol in A4–11 cells showed a 20% reduction in comparison to that in control cells, a 35% decrease was recorded in PY-17 cells. Moreover, when PY-17 cells were rescued by knocking in a rat α1, we found that expression of rat α1 was sufficient to restore both caveolin-1 and cholesterol content (Fig. 1*C*). Finally, when total cellular cholesterol was measured in different cell lines, we found no difference among these cell lines (Fig. 1*D*). These findings suggest that the Na/K-ATPase plays an important role in maintaining the plasma membrane pool of both caveolin-1 and cholesterol in LLC-PK1 cells and that knockdown of the Na/K-ATPase likely redistributes cholesterol from the plasma membrane to other cellular compartments.

Knockdown of the Na/K-ATPase α1 Redistributes Cholesterol from the Plasma Membrane to the Cytosolic Compartments—To further confirm the above observation, we investigated cellular distribution of cholesterol using a fluorescent cholesterol probe, filipin, in both control (P-11) and the knockdown (PY-17) cells. In P-11 cells, a majority of filipin signals (which labels non-esterified cholesterol) were detected in the plasma membrane. Weak signals were also detected in the perinuclear region and in the form of

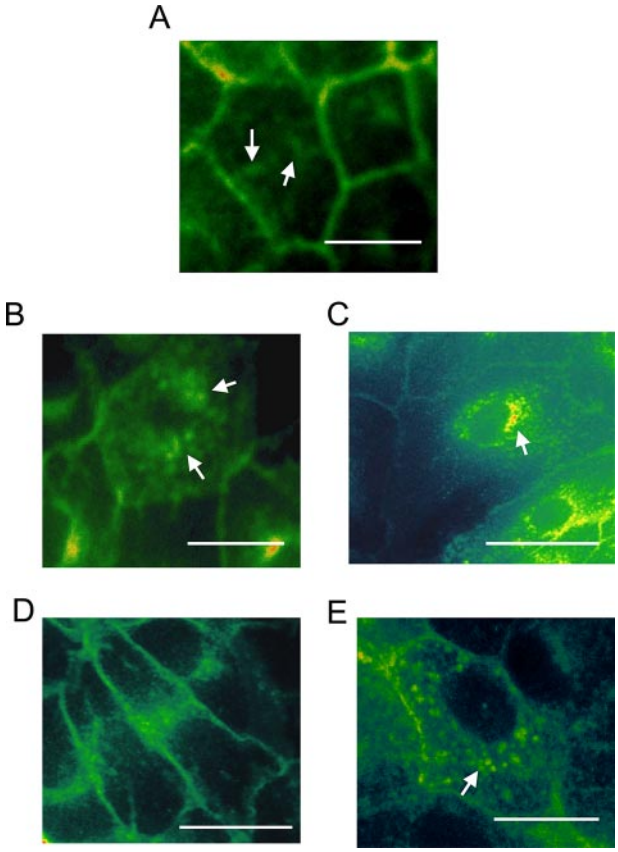

FIGURE 2. **Effects of alterations in Na/K-ATPase or caveolin-1 on cellular cholesterol distribution.** *A*, *B*, *C*, *D*, and *E* show filipin staining of free cholesterol from P-11, PY-17, C2–9, AAC-19, and mCBM cells, respectively. *Arrows* point to the intracellular vesicular filipin signals. *Scale bar*: 20 μm.

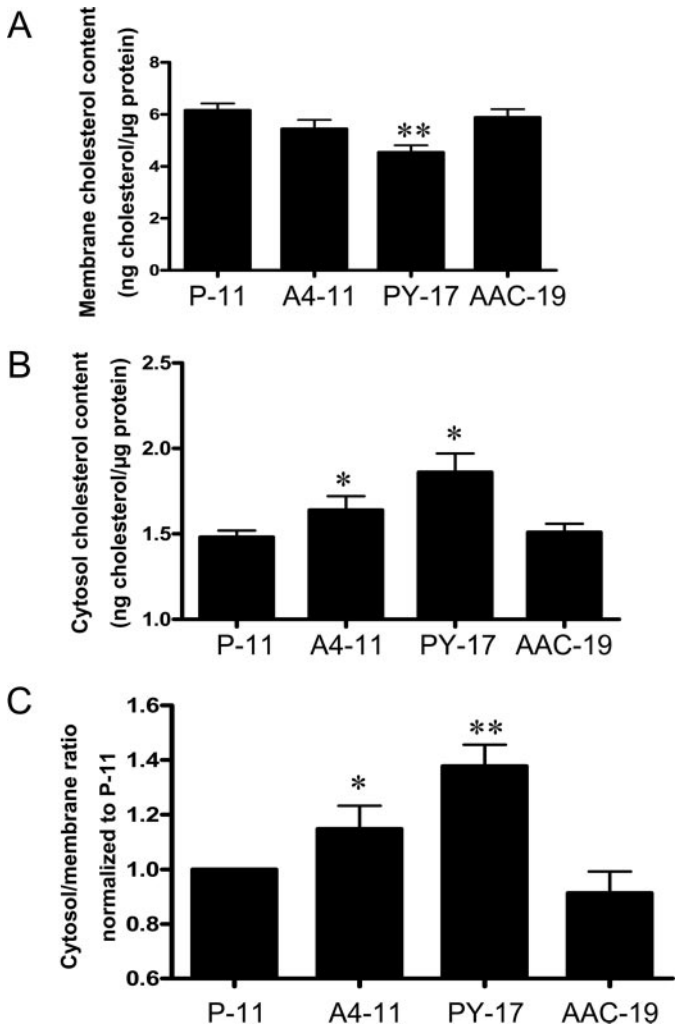

FIGURE 3. **Effects of changes in Na/K-ATPase amount on cytosol and membrane cholesterol content.** Cytosol and membrane fractions were prepared from cell lysates as described under "Experimental Procedures." Cholesterol was measured from the membrane fractions (*A*) and the cytosol fractions (*B*). The ratio between cytosol and membrane cholesterol is also shown (*C*). *, $p < 0.05$ or **, $p < 0.01$ compared with P-11 control cells, $n = 5$.

intracellular vesicles (as indicated by *arrows*) (Fig. 2*A*). These findings are consistent with what has been reported in the literature (31). When compared, PY-17 cells exhibited weaker plasma membrane filipin signals. This is in accordance with the results depicted in Fig. 1. Concomitantly, increases in filipin labeling were noted in the perinuclear region in PY-17 cells. Moreover, PY-17 cells contained many vesicles that were strongly labeled with filipin (as indicated by the *arrows*) (Fig. 2*B*). Taken together, the above studies indicate that knockdown of the Na/K-ATPase redistributed cholesterol from the plasma membrane to intracellular vesicles as well as the perinuclear region.

To test whether cholesterol is redistributed from light caveolar fraction (fraction 4/5) to higher density membrane fractions, we fractionated the cell lysates as in Fig. 1. Afterward, the caveolar fraction (4/5) and non-caveolar fractions (6–11) were diluted and centrifuged at 100,000 $\times$ *g* $\times$ 60 min. The membrane pellets were collected and subjected to cholesterol measurement. Interestingly, no changes in cholesterol content were detected in the combined non-caveolar fractions in the α1 knockdown cells (data not shown). These findings indicate that the plasma membrane cholesterol must be redistributed to cellular compartments (*e.g.* vesicles) that are too light to be pelleted down by 100,000 $\times$ *g* $\times$ 60 min. To further test this postulate, we separated the cell lysates from the control and Na/K-ATPase knockdown cells into two fractions, namely the crude membrane pellet and the cytosol fraction obtained after 100,000 $\times$ *g* $\times$ 60-min centrifugation. When cholesterol was measured in these two fractions, we detected a decrease in cholesterol in the membrane faction (Fig. 3*A*) and a concomitant increase in the cytosol cholesterol (Fig. 3*B*) in cell lysates prepared from the knockdown cells. As expected, the differences in cholesterol distribution among these cells became more obvious when ratios of cytosol over membrane cholesterol were calculated and compared, especially in the case of A4–11 cells (Fig. 3*C*). Consistent with the findings depicted in Fig. 1, knocking in a rat α1 was sufficient to restore the cytosol and membrane cholesterol to a level comparable to that in the control P-11 cells (Fig. 3). Taken together, these studies provided further support to the notion that knockdown of cellular Na/K-ATPase redistributed cholesterol from the plasma membrane to other cellular compartments. The findings also demonstrated that this centrifugation analysis can be used to detect the Na/K-ATPase-mediated cholesterol redistribution.

The Interaction between Na/K-ATPase and Caveolin-1, but Not Src, Is Involved in Control of Plasma Membrane Cholesterol

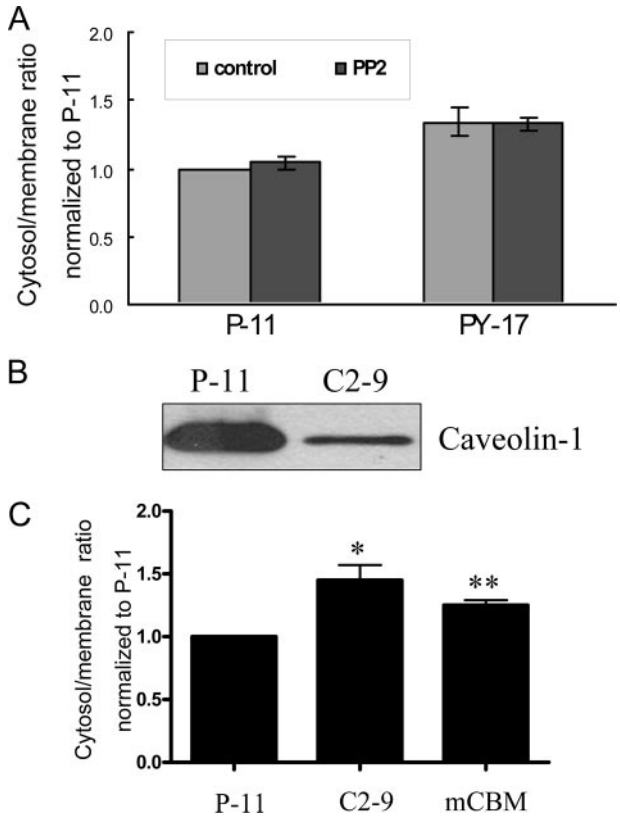

FIGURE 4. **The interaction between the Na/K-ATPase α1 and caveolin-1 is important for proper cholesterol distribution.** *A*, P-11 and PY-17 cells were treated with 1 μM PP2, an Src inhibitor for 2 h. Afterward, cell lysates were fractionated into cytosol and membrane fractions and then subjected to cholesterol measurement. Data are mean ± S.E., $n = 4$. *B*, caveolin-1 protein level was assayed in P-11 and C2–9 cells by Western blot. *C*, cell lysates were processed as in *Panel A* and measured for cholesterol. The cholesterol ratio between cytosol and membrane was calculated. *, $p < 0.05$ or **, $p < 0.01$ compared with P-11 control cells, $n = 3$.

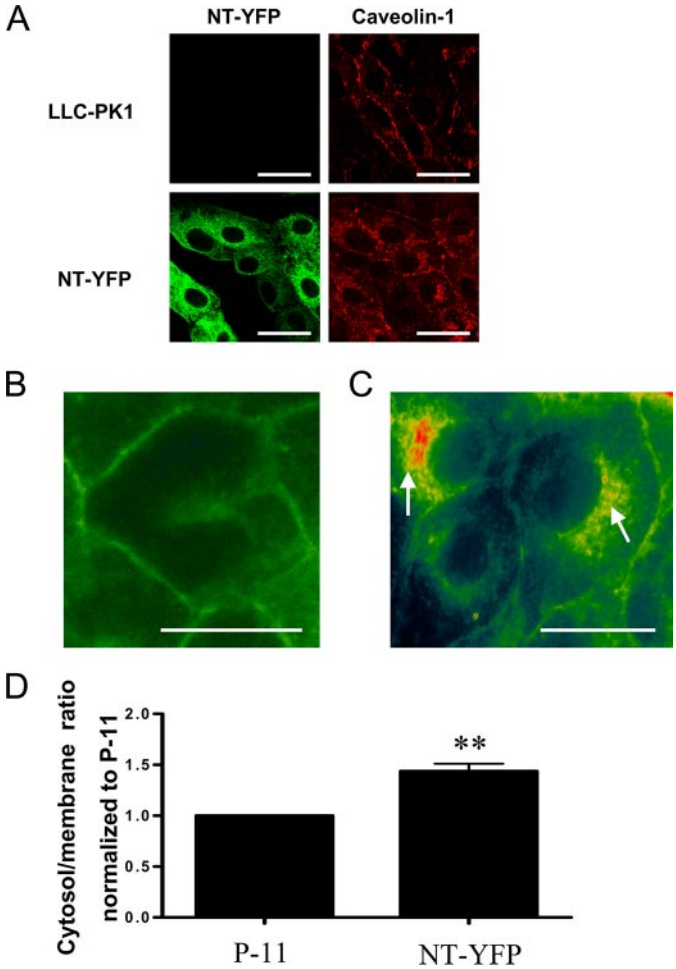

FIGURE 5. **Overexpression of N terminus of Na/K-ATPase α1 redistributes cellular cholesterol.** *A*, a typical confocal image showing the cellular distribution of NT-YFP (*bottom left*) and the endogenous caveolin-1 protein in the LLC-PK1 cells (*top right*) or in the NT-YFP cells (*bottom right*). Immunostaining of caveolin-1 was performed as described under "Experimental Procedures." *B* and *C*, filipin staining of cellular cholesterol from control P-11- and NT-YFP-expressing LLC-PK1 cells. *Scale bar*: 20 μm. *D*, cell lysates were processed as described in Fig. 3, and the cholesterol ratio between cytosol and membrane was calculated. **, $p < 0.01$ compared with P-11 control cells, $n = 3$.

Distribution—The Na/K-ATPase interacts and keeps Src in an inactive state and knockdown of the Na/K-ATPase increases basal Src activity and consequently the activity of ERK1/2 (26). It is known that Src and ERK1/2 play an important role in regulation of cholesterol synthesis (32, 33). Moreover, Src is involved in regulation of caveolin-1 trafficking (16, 34). Therefore, we tested whether inhibition of Src by PP2 would restore the normal cellular distribution of cholesterol. As previously reported, exposure of PY-17 cells to 1 μM PP2 for 2 h was sufficient to restore cellular Src and reduce the mobility of caveolin-1 vesicle (16). However, it failed to change intracellular cholesterol distribution in either control P-11 cells or PY-17 cells when the cytosol/membrane ratios were calculated and compared (Fig. 4*A*).

It is known that caveolin-1 directly binds cholesterol and affects cholesterol trafficking. Because the Na/K-ATPase knockdown changes cellular distribution of caveolin-1, we reasoned that the interaction between the Na/K-ATPase and caveolin-1 could be important for cholesterol trafficking to the plasma membrane. To test this postulation, we first determined the effect of caveolin-1 knockdown on intracellular cholesterol distribution. Caveolin-1 knockdown cell line C2–9 was generated as previously described using small interference RNA (14). As depicted in Fig. 4*B*, these cells express ~20% of caveolin-1 in comparison to that in control P-11 cells. Interestingly, knockdown of caveolin-1, like Na/K-ATPase knockdown, significantly increased the cytosol/membrane ratio of cholesterol (Fig. 4*C*). In accordance, filipin staining showed an accumulation of cytosolic cholesterol vesicles with a concomitant decrease in the plasma membrane cholesterol in C2–9 cells (Fig. 2*C*).

To further test the role of Na/K-ATPase-caveolin-1 interaction, we repeated the above experiments in PY-17 cells rescued by a caveolin-binding motif mutant (mCBM) rat α1. The α1 subunit contains a highly conserved caveolin-binding motif at the N terminus. The mCBM rat α1 was generated by mutating two of the aromatic amino acid residues in the binding motif to Ala (F97A and F100A). We showed previously that these mutations abolished the interaction between the Na/K-ATPase and caveolin-1 (16). As depicted in Fig. 2, expression of mCBM rat α1 (Fig. 2*E*), unlike the expression of wild-type rat α1 (Fig. 2*D*), failed to reduce the vesicular cholesterol staining. Consistently, it also did not restore the cytosol/membrane cholesterol ratio (Fig. 4*C*).

To seek further support and test whether the N terminus of α1 can function as a dominant negative mutant, we transfected

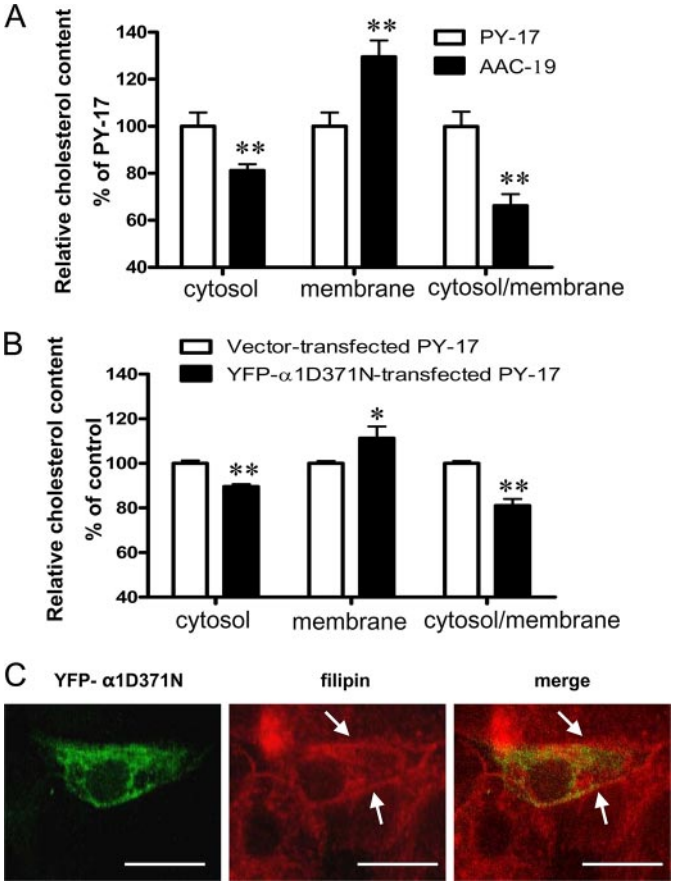

FIGURE 6. **Effects of the expression of α1D371N mutant on cellular cholesterol distribution.** Relative cholesterol content was assayed and calculated in the cytosol and membrane fractions from PY-17 cells and AAC-19 cells, which is wild-type α1-transfected PY-17 cells (*A*) and the vector-transfected and the pump-null D371N transfected PY-17 cells (*B*). *, $p < 0.05$ or **, $p < 0.01$ compared with control cells, $n = 6$. *C*, the representative confocal images of three separate experiments showing (*left panel*) YFP-D371N mutant signal (*middle panel*) filipin signal and (*right panel*) merged image from the pump-null D371N-transfected TCN cells. *Arrows* indicate the plasma membrane signals. *Scale bar*: 20 μm.

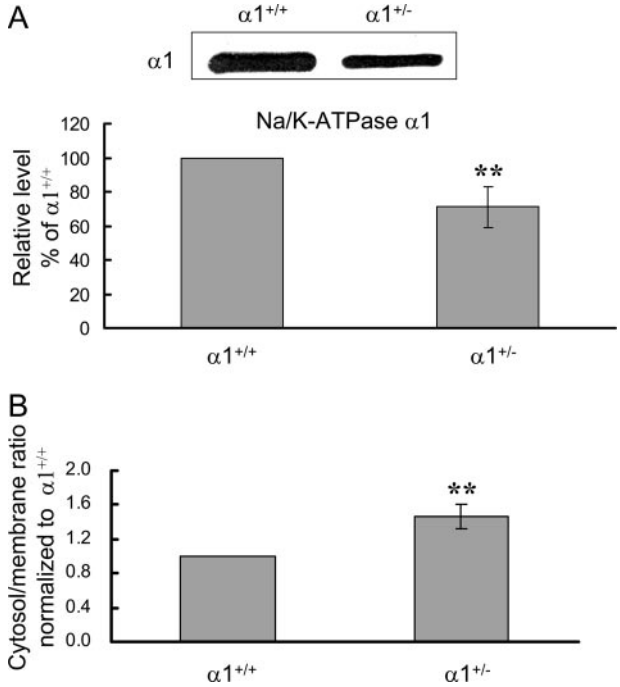

FIGURE 7. **Down-regulation of Na/K-ATPase α1 in mice liver leads to cholesterol redistribution.** *A*, Western blot analysis showed the down-regulation of α1 level in the $\alpha1^{+/-}$ mice liver ($n = 5$). *B*, liver samples from both $\alpha1^{+/+}$ and $\alpha1^{+/-}$ mice were processed as in Fig. 3. Cholesterol from cytosol and membrane fractions was measured, and the ratio was calculated. **, $p < 0.01$ compared with $\alpha1^{+/+}$, $n = 6$.

LLC-PK1 cells with a YFP-tagged N terminus (amino acids 1–160) of α1 (NT-YFP) and generated a stable cell line (27). Confocal imaging analyses revealed that the expressed NT-YFP resided mainly in the intracellular compartments (Fig. 5*A*). Interestingly, expression of NT-YFP led to accumulation of the caveolin-1 protein in the perinuclei regions. Furthermore, filipin staining showed that expression of NT-YFP was sufficient to alter the cholesterol distribution as did by Na/K-ATPase knockdown (Fig. 5, *B* and *C*). Consistently, it also increased cytosol/membrane ratio of cholesterol (Fig. 5*D*).

Expression of a Pump-null α1D371N Mutant Is Capable of Restoring Intracellular Cholesterol Distribution in the Knockdown Cells—To probe whether the pumping function of Na/K-ATPase is required for the regulation of cholesterol distribution, we transfected PY-17 cells with a pump-null mutant (YFP-α1D371N). We showed previously that expression of this mutant was sufficient to restore the plasma membrane distribution of caveolin-1 (16). Consistently, we found that, like YFP-α1, expression of YFP-α1D371N mutant in PY-17 decreased the cytosol cholesterol and concomitantly increased the membrane cholesterol, resulting in significant reduction in the ratio of cytosol/membrane cholesterol (Fig. 6, *A* and *B*). It is important to note that these measurements were made in transiently transfected PY-17 cells, because we could not generate stable cell lines that express YFP-α1D371N. To seek further evidence, we transfected TCN23-19 cells with the same mutant construct and then stained transfected cells with filipin. Like PY-17 cells, TCN23-19 cells express <10% of Na/K-ATPase (26). Unlike PY-17 cells, these cells do not express green fluorescent protein, making it easier to conduct imaging analyses of YFP-α1D371N distribution. As shown in Fig. 6*C*, the plasma membrane expression of the pump-null D371N mutant was able to increase the filipin signal in the plasma membrane. However, because of low transfection efficiency, this increase in the plasma membrane cholesterol appeared to be modest. Taken together, these findings indicate that the Na/K-ATPase can regulate cholesterol distribution independent from its pumping function.

Down-regulation of Na/K-ATPase α1 Subunit in Mice Leads to Hepatic Cholesterol Redistribution—The data from LLC-PK1 cells indicated that the Na/K-ATPase α1 interacts with caveolin-1 and plays an important role in regulating intracellular cholesterol distribution. To further test the physiological relevance of the above findings, we determined whether reduction of the α1 expression in $\alpha1^{+/-}$ mice could alter cellular cholesterol distribution. Because liver plays an essential role in the cholesterol metabolism, here we focused on liver tissue samples from $\alpha1^{+/+}$ and $\alpha1^{+/-}$ mice. As illustrated in Fig. 7*A*, Western blot analysis showed a 30% decrease in α1 expression in the $\alpha1^{+/-}$ livers, which was consistent with previous report on other tissues (3). When cholesterol was measured, we

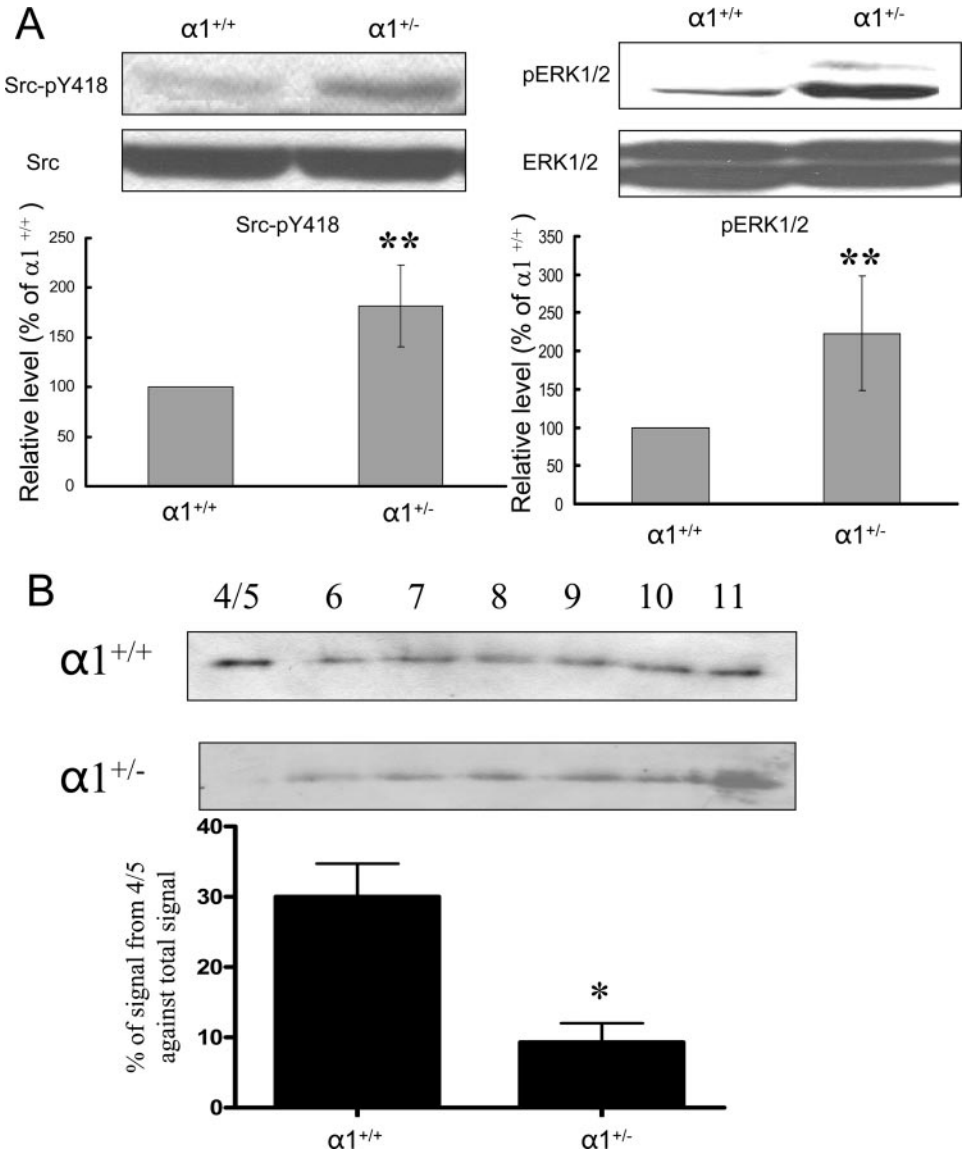

FIGURE 8. **Down-regulation of Na/K-ATPase α1 in mouse liver leads to the activation of Src and ERK1/2 and redistribution of caveolin-1.** *A*, Western blot analysis showed the increase in active Src (Src-pY418) and pERK1/2 in $\alpha1^{+/-}$ mice liver samples. The quantitative data are presented as mean ± S.E. $n = 6$. *B*, liver samples from $\alpha1^{+/+}$ mice and $\alpha1^{+/-}$ mice were subjected to sucrose density fractionation as in Fig. 1. Caveolin-enriched fractions (4/5) together with fractions 6~11 were taken for Western blot analysis of caveolin-1. A representative Western blot from three independent experiments is shown. The percentage of signals from caveolin-enriched fractions against total signals is shown below. *, $p < 0.05$ compared with $\alpha1^{+/+}$.

observed that reduction of the α1 expression in liver significantly increased the cytosol/membrane ratio of cholesterol (Fig. 7*B*), which is consistent with the data obtained in cultured cells (Fig. 3).

To be sure that reduction in the cellular amount of Na/K-ATPase alters the interaction among the Na/K-ATPase and its partners *in vivo*, we measured cellular Src and ERK activity as well as caveolin-1 distribution. As shown in Fig. 8*A*, we detected a significant increase in cellular Src and ERK1/2 activity in $\alpha1^{+/-}$ liver samples. Moreover, knockdown of the Na/K-ATPase significantly redistributed caveolin-1 from fraction 4/5 to high density fractions in liver tissues as detected in cultured cells (Fig. 8*B*) (16).

Taken together, the above *in vitro* and *in vivo* studies indicate that the Na/K-ATPase plays a role in regulation of cellular cholesterol distribution. Knockdown of the Na/K-ATPase could reduce the plasma membrane pool of cholesterol, resulting in a concomitant increase in cholesterol in the "cytosol."

Reduction of Cellular Na/K-ATPase Activates SREBP2, Resulting in an Increase in Cellular HMG-CoA Reductase and LDL Receptor in Vivo—It is known that the ER cholesterol is highly regulated by the plasma membrane cholesterol (35). Therefore, it is conceivable that the Na/K-ATPase knockdown-induced reduction in the plasma membrane cholesterol could alter the ER cholesterol and then the activity of SREBP2, a cholesterol-sensitive transcription factor (36). These studies were performed on liver samples from both $\alpha1^{+/+}$ and $\alpha1^{+/-}$ mice, because our preliminary studies failed to detect SREBP2 using the commercially available antibodies in LLC-PK1 cells. As depicted in Fig. 9*A*, we observed a 40% increase in the amount of active SREBP2 in liver samples from the $\alpha1^{+/-}$ mice, supporting the notion that knockdown of the α1 may be sufficient to alter the ER cholesterol sensing process. To seek additional support, we measured the amount of HMG-CoA reductase in the liver samples. As shown in Fig. 9*B*, a 100% increase in HMG-CoA reductase was recorded in $\alpha1^{+/-}$ mouse liver. Increases in the amount of HMG-CoA reductase could be the result of two ER cholesterol-mediated regulations. Although a decrease in the ER cholesterol reduces the degradation of HMG-CoA reductase (37), activation of the SREBP pathway increases the expression of the enzyme. Thus, to determine whether increases in the active form of SREBP2 stimulate the expression of SREBP2-responsible genes, we also measured the cellular amount of LDL receptor. Consistently, there was also a 40% increase in cellular LDL receptor in livers from $\alpha1^{+/-}$ mice (Fig. 9*C*).

A Reduction in Cellular Na/K-ATPase Is Sufficient to Change Liver Cholesterol Metabolism and Affect Plasma Lipid Profile—To further explore the significance of Na/K-ATPase in cholesterol metabolism, we tested whether increases in cellular HMG-CoA reductase and LDL receptor affect total cellular cholesterol. As depicted in Table 2, we found a 15% increase in total cellular cholesterol in livers from $\alpha1^{+/-}$ mice. Consis-

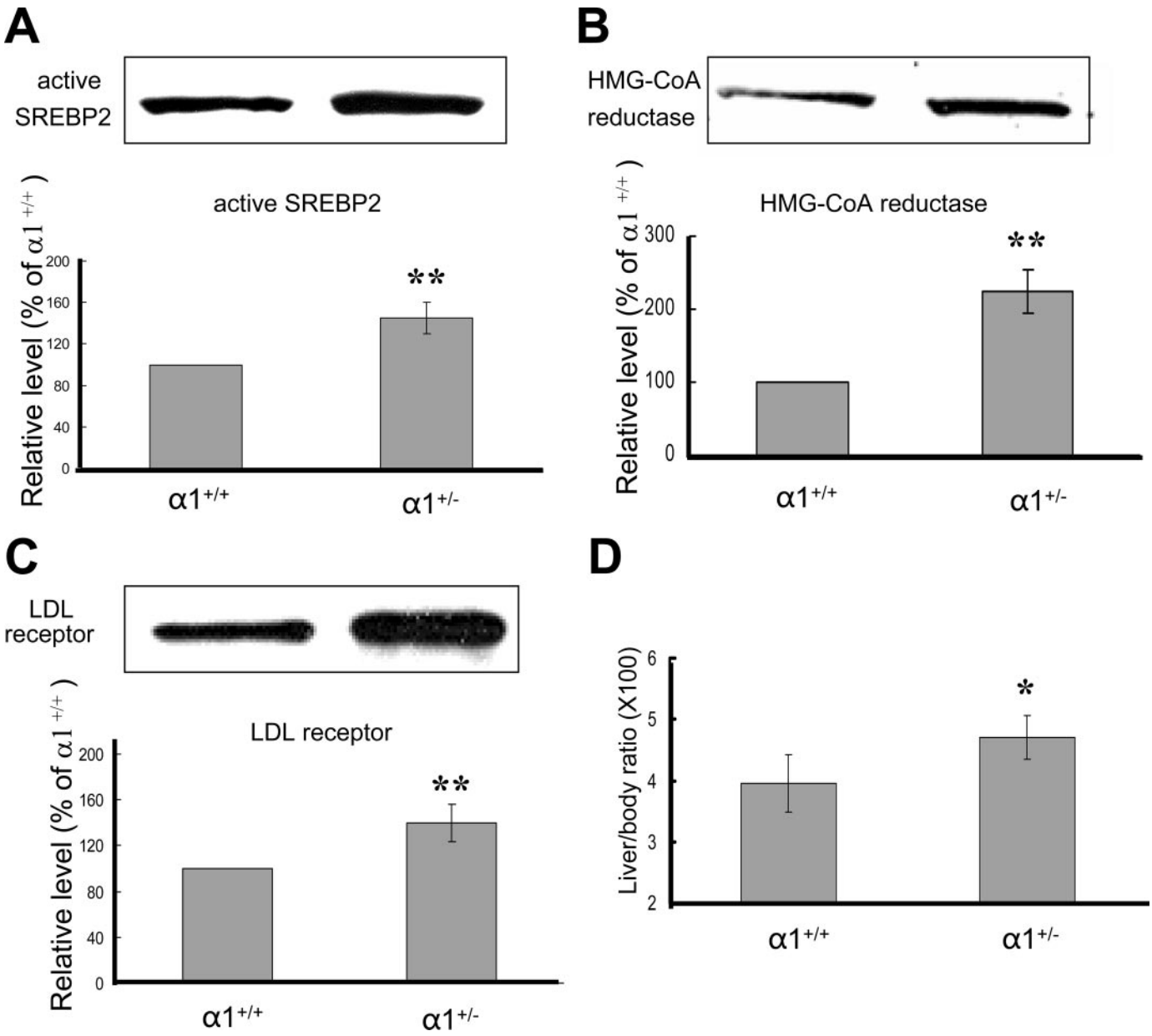

FIGURE 9. **SREBP2 pathway is activated in $\alpha 1^{+/-}$ mice liver.** *A–C*, samples from both $\alpha 1^{+/+}$ and $\alpha 1^{+/-}$ mice livers were analyzed by Western blot for the active form of SREBP2, HMG-CoA reductase, and LDL receptor, respectively. A representative Western is shown, and quantitative data are collected from 5 $\alpha 1^{+/+}$ and 5 $\alpha 1^{+/-}$ mice livers. *D*, the liver weight/body weight ratio was calculated from four $\alpha 1^{+/-}$ mice and seven $\alpha 1^{+/+}$ mice livers. *, $p < 0.05$, **, $p < 0.01$ compared with $\alpha 1^{+/+}$.

TABLE 2

Down-regulation of $\alpha 1$ affects liver cholesterol content and plasma lipid profile

	$\alpha 1^{+/+}$	$\alpha 1^{+/-}$
Cholesterol/unit of protein (% of WT)	100 ± 2	115 ± 5[a]
Plasma cholesterol (mg/dl)	120 ± 11.1	81.7 ± 25.8[b]

[a] $p < 0.05$ compared to $\alpha 1^{+/+}$.
[b] $p < 0.01$ compared to $\alpha 1^{+/+}$.

tently, when plasma cholesterol was measured, plasma cholesterol content was significantly decreased in the $\alpha 1^{+/-}$ mice. Moreover, the livers from the $\alpha 1^{+/-}$ mice appeared to be larger (Fig. 9*D*). Taken together, these data indicate that reduction of the Na/K-ATPase redistributes hepatic cholesterol, which subsequently alters cholesterol metabolism *in vivo*.

DISCUSSION

In this study, we have demonstrated that the Na/K-ATPase regulates intracellular cholesterol distribution via its interaction with caveolin-1. This takes place not only in cultured cells but also in intact animals. We have further revealed that cholesterol redistribution in the $\alpha 1^{+/-}$ mouse liver is accompanied by alteration of cholesterol metabolism. Taken together, these results strongly suggest that the Na/K-ATPase that pumps and signals may also play a role in maintaining cholesterol pool in the plasma membrane, thus providing a sensing mechanism for cells to regulate cellular cholesterol metabolism.

Regulation of Intracellular Cholesterol Distribution by the Na/K-ATPase—We have recently suggested that there is a large pool of non-pumping Na/K-ATPase in LLC-PK1 cells. It

appears that a large portion of the non-pumping Na/K-ATPase resides in caveolae and dynamically interacts with various proteins, including caveolin-1 and Src (14). Moreover, we found that reduction of this pool of Na/K-ATPase mobilized caveolin-1, resulting in a decrease in plasma membrane caveolin-1 and a concomitant accumulation of caveolin-1 in intracellular vesicles (16). Here, we have further established that knockdown of cellular Na/K-ATPase also reduced the amount of cholesterol in the plasma membrane. Filipin labeling indicated that the plasma membrane cholesterol was redistributed to intracellular compartments in the knockdown cells (Fig. 2*B*). When total cell lysates were separated into membrane and cytosol pools, we found that the membrane cholesterol was redistributed to the cytosol fractions in the α1 knockdown cells. Moreover, this redistribution effect was correlated to the amount of cellular Na/K-ATPase (Fig. 3). This increase was reversible. Expression of an exogenous rat α1 restored not only cellular Na/K-ATPase, but also cholesterol distribution in PY-17 cells (Fig. 3). Furthermore, it appears that the effect of Na/K-ATPase on cellular cholesterol distribution is independent of its pumping activity, because expression of a pump-null mutant α1 (D371N) was capable of restoring membrane distribution of cholesterol in the knockdown PY-17 cells (Fig. 6). Consistently, we found that A4–11 cells exhibit similar pumping activity as that of PY-17 cells, but much less change in cholesterol distribution (Fig. 3) (11). Finally, similar changes were detected in livers from $\alpha1^{+/-}$ mice (Figs. 7 and 8). Knockdown of the Na/K-ATPase α1 not only increased basal Src and ERK1/2 activity, but also altered caveolin-1 and cholesterol distribution.

Changes in cellular cholesterol distribution have been reported (38–40). The most notable is the case of Niemann-Pick type C1 (NPC1) disease, which is characterized by a massive accumulation of cholesterol in cytoplasmic vacuoles. These structures resemble lysosomes/late endosomes, and are of low buoyant density (41). Similar accumulation has also been observed when a truncated caveolin-3 mutant was expressed in epithelial cells (39). Moreover, accumulation of cholesterol in late endosomes occurred when cells were exposed to drug U18666A. Interestingly, these alterations appear to be caused by the accumulation of cholesterol in late endosomes, which is probably due to a defect of the cholesterol trafficking between plasma membrane and late endosomes (38). We showed that, in the Na/K-ATPase knockdown cells, filipin labeling of intracellular vesicles was clearly increased (Fig. 2*B*). In addition, these vesicles are too buoyant to be centrifuged down at 100,000 $\times$ *g* $\times$ 60 min. Thus, it is tempting to suggest that the Na/K-ATPase knockdown produced a similar change in cellular cholesterol distribution as in NPC1 cells or in cells exposed to U18666A. Alternatively, it is known that there is a pool of soluble caveolin/cholesterol complexes that appear to play a role in intracellular cholesterol trafficking (22).

The Interaction between Na/K-ATPase and Caveolin-1 Is Important for Maintaining the Plasma Membrane Pool of Cholesterol—Na/K-ATPase is known to interact with caveolin-1 and Src directly (14). Moreover, these interactions play a pivotal role in regulation of caveolin-1 trafficking (16). Finally, it is known that caveolin-1 directly binds cholesterol in a 1:1 ratio (21) and that caveolin-1 regulates cholesterol trafficking from and to the plasma membrane (22, 23). Thus, the Na/K-ATPase knockdown could reduce caveolin-1 and consequently cholesterol in the plasma membrane. The notion appears to be supported by our new findings presented in Figs. 2–8. First, knockdown of caveolin-1 was sufficient to alter cholesterol distribution as did the Na/K-ATPase knockdown. Second, expression of a wild-type rat α1, but not a CBM mutant rat α1, could restore cholesterol distribution in PY-17 cells. Finally, overexpression of the N terminus of α1 subunit that contains the caveolin-binding motif was equally effective in redistributing cholesterol from the plasma membrane to intracellular vesicles. However, other alternatives should also be noted. It is known that cholesterol affects both the pumping and signaling function of Na/K-ATPase (11, 14, 42–44). Several of these early studies suggested that cholesterol directly interacts with the Na/K-ATPase. Interestingly, when we searched the α1 for a potential cholesterol binding consensus (-(L/V)X_{1-5}YX_{1-5}(R/K)-) (45), we found three potential binding sites, one at the first transmembrane domain and two at the last transmembrane domain. Moreover, they are highly conserved within mammals and even toad and fish. Thus, it is plausible that the potential interaction between the Na/K-ATPase and cholesterol may also play a role in regulation of cholesterol distribution. Needless to say, this issue remains to be resolved.

TABLE 3
Comparison between NPC1 knockout mice and $\alpha1^{+/-}$ mice shows many similarities in terms of the alteration of cholesterol metabolism

	NPC1$^{-/-a}$	$\alpha1^{+/-}$
Body weight	No change	No change
Liver weight/body weight	Little ↑ but not significant	Little increase
Liver cholesterol content	↑	↑
Total plasma cholesterol	↓	↓
SREBP2	↑ (mRNA)	↑ (protein)

[a] ↑, increase; ↓, decrease.

Regulation of Cholesterol Sensing by the Na/K-ATPase in Vivo—Liver plays an essential role in cholesterol metabolism. It is one of the major organs for *de novo* cholesterol biosynthesis and is the predominant site for LDL uptake via LDL receptor-mediated endocytosis, which is a major factor for lowering the plasma LDL level (46). Generally, hepatic cholesterol level is tightly regulated by the cellular cholesterol sensing mechanism. Lowering the ER cholesterol will reduce the binding of cholesterol to SCAP, which will activate the transcription factor SREBP2 and prolong the half-life of HMG-CoA reductase (47). Activation of SREBP2 can also increase the transcription of HMG-CoA reductase and LDL receptor. It is known that most of cellular cholesterol resides in the plasma membrane and that the ER cholesterol is highly sensitive to the plasma membrane cholesterol (35). Thus, it is conceivable that lowering the plasma membrane cholesterol by knockdown of the Na/K-ATPase may abolish the cholesterol transport from the plasma membrane to the ER, resulting in a reduction in the ER cholesterol and subsequent activation of SREBP2. Consistently, our results showed an activation of the SREBP2 pathway in $\alpha1^{+/-}$ mouse livers. This notion is further supported by increases in both HMG-CoA reductase and LDL receptor (Fig. 9). Interestingly, as illustrated in Table 3, changes in cholesterol metabo-

lism detected in $\alpha1^{+/-}$ mice share many similarities with those found in NPC1$^{-/-}$ mice (48). NPC1 knockout caused significant activation of SREBP2, resulting in an increase in hepatic cholesterol and a decrease in plasma cholesterol (48). As expected, the $\alpha1^{+/-}$ and SREBP2 transgenic mice also share many similarities (36). Taken together, our data suggest that reduction of cellular Na/K-ATPase may decrease the ER cholesterol and subsequently alter cellular cholesterol metabolism via the activation of a SREBP2-dependent pathway. These findings are significant. First, to our knowledge, this is the first report showing the involvement of Na/K-ATPase in control of cholesterol sensing. Second, the data suggest that maintaining a stable pool of Na/K-ATPase is important for controlling the ER cholesterol and thus cholesterol metabolism. To this end, it is of interest to mention that the expression of Na/K-ATPase is significantly reduced in metabolic diseases such as atherosclerosis (49), obesity (50), and type 2 diabetes (51, 52). Thus, our new findings warrant further investigation of the role of Na/K-ATPase in regulation of cholesterol metabolism.

Acknowledgments—We thank Manoranjani P. M. Tillekeratne for excellent technical support; Dr. David R. Giovannucci, Dr. Christian G. Peters, and Dr. Andrea Kalinoski for help with the imaging study; and Matha Heck for editing the manuscript.

REFERENCES

1. Skou, J. C. (1957) *Biochim. Biophys. Acta* **23,** 394–401
2. Lingrel, J. B., and Kuntzweiler, T. (1994) *J. Biol. Chem.* **269,** 19659–19662
3. James, P. F., Grupp, I. L., Grupp, G., Woo, A. L., Askew, G. R., Croyle, M. L., Walsh, R. A., and Lingrel, J. B. (1999) *Mol. Cell* **3,** 555–563
4. Sweadner, K. J. (1989) *Biochim. Biophys. Acta* **988,** 185–220
5. Lingrel, J. B., Orlowski, J., Shull, M. M., and Price, E. M. (1990) *Prog. Nucleic Acid Res. Mol. Biol.* **38,** 37–89
6. Kometiani, P., Li, J., Gnudi, L., Kahn, B. B., Askari, A., and Xie, Z. (1998) *J. Biol. Chem.* **273,** 15249–15256
7. Xie, Z., Kometiani, P., Liu, J., Li, J., Shapiro, J. I., and Askari, A. (1999) *J. Biol. Chem.* **274,** 19323–19328
8. Li, S., and Wattenberg, E. V. (1998) *Toxicol. Appl. Pharmacol.* **151,** 377–384
9. Xie, Z. (2001) *Cell. Mol. Biol.* **47,** 383–390
10. Haas, M., Wang, H., Tian, J., and Xie, Z. (2002) *J. Biol. Chem.* **277,** 18694–18702
11. Liang, M., Tian, J., Liu, L., Pierre, S., Liu, J., Shapiro, J., and Xie, Z. J. (2007) *J. Biol. Chem.* **282,** 10585–10593
12. Tian, J., Cai, T., Yuan, Z., Wang, H., Liu, L., Haas, M., Maksimova, E., Huang, X. Y., and Xie, Z. J. (2006) *Mol. Biol. Cell* **17,** 317–326
13. Yuan, Z., Cai, T., Tian, J., Ivanov, A. V., Giovannucci, D. R., and Xie, Z. (2005) *Mol. Biol. Cell* **16,** 4034–4045
14. Wang, H., Haas, M., Liang, M., Cai, T., Tian, J., Li, S., and Xie, Z. (2004) *J. Biol. Chem.* **279,** 17250–17259
15. Haas, M., Askari, A., and Xie, Z. (2000) *J. Biol. Chem.* **275,** 27832–27837
16. Cai, T., Wang, H., Chen, Y., Liu, L., Gunning, W. T., Quintas, L. E., and Xie, Z. J. (2008) *J. Cell Biol.* **182,** 1153–1169
17. Williams, T. M., and Lisanti, M. P. (2004) *Genome Biol.* **5,** 214
18. Fielding, C. J., and Fielding, P. E. (2000) *Biochim. Biophys. Acta* **1529,** 210–222
19. Parton, R. G., Hanzal-Bayer, M., and Hancock, J. F. (2006) *J. Cell Sci.* **119,** 787–796
20. Razani, B., Woodman, S. E., and Lisanti, M. P. (2002) *Pharmacol. Rev.* **54,** 431–467
21. Murata, M., Peranen, J., Schreiner, R., Wieland, F., Kurzchalia, T. V., and Simons, K. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92,** 10339–10343
22. Uittenbogaard, A., Ying, Y., and Smart, E. J. (1998) *J. Biol. Chem.* **273,** 6525–6532
23. Uittenbogaard, A., Everson, W. V., Matveev, S. V., and Smart, E. J. (2002) *J. Biol. Chem.* **277,** 4925–4931
24. Bist, A., Fielding, P. E., and Fielding, C. J. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94,** 10693–10698
25. Frank, P. G., Cheung, M. W., Pavlides, S., Llaverias, G., Park, D. S., and Lisanti, M. P. (2006) *Am. J. Physiol. Heart Circ. Physiol.* **291,** H677–686
26. Liang, M., Cai, T., Tian, J., Qu, W., and Xie, Z. J. (2006) *J. Biol. Chem.* **281,** 19709–19719
27. Chen, Y., Cai, T., Yang, C., Turner, D. A., Giovannucci, D. R., and Xie, Z. (2008) *J. Biol. Chem.* **283,** 1128–1136
28. Carozzi, A. J., Ikonen, E., Lindsay, M. R., and Parton, R. G. (2000) *Traffic* **1,** 326–341
29. Liu, L., Mohammadi, K., Aynafshar, B., Wang, H., Li, D., Liu, J., Ivanov, A. V., Xie, Z., and Askari, A. (2003) *Am. J. Physiol. Cell Physiol.* **284,** C1550–1560
30. Sampson, L. J., Hayabuchi, Y., Standen, N. B., and Dart, C. (2004) *Circ. Res.* **95,** 1012–1018
31. Lange, Y., Swaisgood, M. H., Ramos, B. V., and Steck, T. L. (1989) *J. Biol. Chem.* **264,** 3786–3793
32. Sun, Y., Ma, Y. C., Huang, J., Chen, K. Y., McGarrigle, D. K., and Huang, X. Y. (2005) *Biochemistry* **44,** 14455–14462
33. Abidi, P., Zhang, F., Li, C., and Liu, J. (2005) *Int. J. Mol. Med.* **16,** 779–785
34. Pelkmans, L., and Zerial, M. (2005) *Nature* **436,** 128–133
35. Lange, Y., Ye, J., Rigney, M., and Steck, T. L. (1999) *J. Lipid Res.* **40,** 2264–2270
36. Horton, J. D., Shimomura, I., Brown, M. S., Hammer, R. E., Goldstein, J. L., and Shimano, H. (1998) *J. Clin. Invest.* **101,** 2331–2339
37. Goldstein, J. L., DeBose-Boyd, R. A., and Brown, M. S. (2006) *Cell* **124,** 35–46
38. Lange, Y., Ye, J., Rigney, M., and Steck, T. (2000) *J. Biol. Chem.* **275,** 17468–17475
39. Pol, A., Luetterforst, R., Lindsay, M., Heino, S., Ikonen, E., and Parton, R. G. (2001) *J. Cell Biol.* **152,** 1057–1070
40. Puri, V., Jefferson, J. R., Singh, R. D., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (2003) *J. Biol. Chem.* **278,** 20961–20970
41. Sobo, K., Le Blanc, I., Luyet, P. P., Fivaz, M., Ferguson, C., Parton, R. G., Gruenberg, J., and van der Goot, F. G. (2007) *PLoS ONE* **2,** e851
42. Yeagle, P. L. (1983) *Biochim. Biophys. Acta* **727,** 39–44
43. Giraud, F., Claret, M., and Garay, R. (1976) *Nature* **264,** 646–648
44. Cornelius, F. (1995) *Biochim. Biophys. Acta* **1235,** 205–212
45. Li, H., and Papadopoulos, V. (1998) *Endocrinology* **139,** 4991–4997
46. Brown, M. S., and Goldstein, J. L. (1986) *Science* **232,** 34–47
47. Brown, M. S., and Goldstein, J. L. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96,** 11041–11048
48. Garver, W. S., Jelinek, D., Oyarzo, J. N., Flynn, J., Zuckerman, M., Krishnan, K., Chung, B. H., and Heidenreich, R. A. (2007) *J. Cell. Biochem.* **101,** 498–516
49. Chen, M., Mason, R. P., and Tulenko, T. N. (1995) *Biochim. Biophys. Acta* **1272,** 101–112
50. De Luise, M., Blackburn, G. L., and Flier, J. S. (1980) *N. Engl. J. Med.* **303,** 1017–1022
51. Djurhuus, M. S., Vaag, A., and Klitgaard, N. A. (2001) *J. Clin. Endocrinol. Metab.* **86,** 859–866
52. Noda, K., Umeda, F., Hashimoto, T., Yamashita, T., and Nawata, H. (1990) *Diabetes Res. Clin. Pract.* **8,** 177–181