Silencing of the mutant SCAP allele accounts for restoration of a normal phenotype in CT60 cells selected for NPC1 expression

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Abstract **The sterol regulatory element binding protein (SREBP)/SREBP cleavage-activating protein (SCAP) complex regulates the transcription of numerous genes involved in cellular cholesterol metabolism. The CHO mutant, CT60, and its parental cell line, 25RA, possess a gain-of-function mutation in one allele of the SCAP gene that renders the cells resistant to sterol-mediated suppression of cholesterol synthesis and uptake. In addition, CT60 cells do not express a functional Niemann-Pick type C1 (NPC1) protein, which leads to lysosomal accumulation of free cholesterol. Correction of the NPC1 defect by expression of a yeast artificial chromosome (YAC) containing the NPC1 genetic interval restored normal mobilization of cholesterol from the lysosomal compartment. Unexpectedly, the YAC-containing cell lines have overall cellular cholesterol concentrations that are comparable to wild-type levels, despite the assumed presence of the SCAP mutation. This phenotypic change results from a reduction in endogenous sterol synthesis, LDL receptor message, and HMG-CoA reductase message. Genetic analysis of the SCAP gene revealed that the YAC-expressing CT60 cells have normal regulation of these sentinel cholesterogenic genes as a result of selective silencing of the mutant SCAP allele, which appears to be independent of functional NPC1 expression.**—Maguire, J. A., and J. W. Reagan, Jr. **Silencing of the mutant SCAP allele accounts for restoration of a normal phenotype in CT60 cells selected for NPC1 expression.** ***J. Lipid Res.*** **2005.** 46: **1840–1848.**

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CHO cells are pseudodiploid, and many genes in these cells are inactivated by epimutations such as methylation, which do not alter the DNA sequence but cause certain genes to be functionally haploid (1–3). These traits make CHO cells more amenable to genetic manipulation than other cell lines, providing an ideal model for mutational studies (4). A number of CHO mutants defective in cholesterol metabolism have been generated and used to identify key regulatory components of intracellular cholesterol metabolic pathways (3), including the sterol regulatory element binding protein (SREBP)/SREBP cleavage-activating protein (SCAP) pathway (5). SREBPs are membrane-bound transcription factors that control the transcription of genes involved in lipid synthesis and lipoprotein uptake into the cell, including HMG-CoA reductase and the LDL receptor, respectively (6–8). Through an intricate pathway, the SREBP protein is escorted from the endoplasmic reticulum (ER) to the Golgi complex by SCAP, an integral membrane protein whose function is regulated by cholesterol concentrations in the ER (5, 9). This translocation results in proteolytic cleavage of the SREBP N-terminal transcription factor within the Golgi, which subsequently relocates to the nucleus to upregulate specific genes (10–14). The ability of SCAP to detect the concentration of cholesterol in the ER is mediated through a sterol-sensing domain (SSD), a 180 amino acid membrane-spanning region: low sterol concentrations promote the movement of the protein complex and the formation of the transcription factor, whereas high concentrations promote the binding of a retention protein to SCAP that inhibits translocation and the formation of the transcription factor (9, 15–17).

25RA cells were one of the mutant lines used to elucidate this pathway and were obtained by chemical mutagenesis of wild-type CHO cells using ethyl methanesulfonate (18). These cells possess a point mutation at codon 443 of the SCAP gene, which transforms an aspartic acid to an asparagine within the SSD, eliminating the sterol-dependent

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Abbreviations: ER, endoplasmic reticulum; NPC1, Niemann-Pick type C1; SCAP, sterol regulatory element binding protein cleavage-activating protein; SREBP, sterol regulatory element binding protein; SSD, sterol-sensing domain; YAC, yeast artificial chromosome.

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retention of SREBP in the ER (19). The mutation does not affect the binding of cholesterol to SCAP but disrupts the interaction between SCAP and the retention protein (16, 20). Therefore, increased concentrations of sterols fail to induce the retention of SREBP, promoting the formation of the transcription factor and the upregulation of gene expression regardless of sterol concentrations. This cell line has proven extremely valuable not only in the identification of the SREBP/SCAP pathway (13, 19) but also in the characterization of ACAT (21) and the Niemann-Pick type C1 (NPC1) protein (22).

25RA cells were further mutagenized with *N*-nitroso-*N*-ethylurea, resulting in three CT (cholesterol-trafficking) clones, one of which was the CT60 cell line studied here (4). In addition to possessing the aforementioned SCAP mutation, CT60 cells exhibit gross accumulation of free cholesterol in the lysosomal compartment. This phenotype results from a defect in the late endosomal NPC1 protein, which participates in the intracellular trafficking of LDL-derived and endogenously synthesized cholesterol from the late endosomal/lysosomal system to various intracellular locations (23–26). Further analysis demonstrated that the CT60 cell line is genetically comparable to human and mouse NPC cell lines; therefore, it was used to evaluate the ability of a 590 kb yeast artificial chromosome (YAC) containing the putative NPC1 genetic interval to complement the mutation (27). Expression of the genes contained in this YAC corrected the mutation in the CT60 cells, resulting in the mobilization of lysosomal cholesterol, thus identifying the NPC1 gene and demonstrating its role in intracellular cholesterol transport (22, 27).

Theoretically, correction of the NPC1 defect in CT60 cells by expression of the NPC1-containing YAC should result in the restoration of the 25RA phenotype. However, preliminary experiments indicated that the YAC-expressing cell lines possessed a normal phenotype rather than the expected parental 25RA phenotype. This suggested a potential interaction between NPC1 and the SREBP/SCAP pathway. In fact, Ory and colleagues (28) demonstrated that overexpression of NPC1 results in resistance to the sterol-induced inhibition of SREBP cleavage. Such an interaction could be facilitated by the SSD, which is highly conserved in a number of proteins, including NPC1, HMG-CoA reductase, and SCAP (29). The SSD of NPC1 may compete with SCAP for binding to INSIG, as described for HMG-CoA reductase (30). This would facilitate the sterol-independent movement of the SREBP/SCAP complex to the Golgi, with subsequent generation of the active SREBP transcription factor. Identical mutations in the NPC1 and SCAP SSDs induce a gain of function in both proteins, providing further support for the role of the NPC1 SSD in SCAP regulation (31).

To determine whether such an interaction exists, we studied the effect of NPC1-YAC expression on cholesterol homeostasis in the NPC1-defective background of CT60 cells (27). Although our cholesterol metabolism experiments were consistent with a potential relationship between NPC1 and the SREBP/SCAP pathway, it was subsequently demonstrated that restoration to a normal phenotype resulted from apparent silencing of the SCAP mutation. The mechanism by which this gene modification occurs is unknown, but it is not believed to result from the expression or function of NPC1.

MATERIALS AND METHODS

Materials

Wild-type CHO cells were purchased from the American Type Culture Collection (Manassas, VA). CT60 and 25RA cells were kindly provided by Dr. T. Y. Chang (Dartmouth Medical School, Hanover, NH). CT60-HN, CT60-A1, and CT60-A13 cells were kindly provided by Dr. Peter Pentchev (National Institutes of Health, Bethesda, MD). CT60 cells stably expressing mouse NPC1-EYFP were kindly provided by Dr. Matthew Scott (Stanford University, Stanford, CA) (25). Tissue culture media and supplements were obtained from Mediatech (Herndon, VA). FBS was obtained from Atlantic Biologicals (Norcross, GA). All tissue culture plasticware was obtained from Corning (Corning, NY). Trypsin was obtained from JRH Biosciences (Lenexa, KS). Trizol and geneticin were obtained from Invitrogen (Carlsbad, CA). Primers were obtained from IDT DNA Technologies. PCR Master Mix was obtained from Promega (Madison, WI). The Omniscript Reverse Transcription Kit was obtained from Qiagen (Valencia, CA). SYBR green Master Mix was obtained from Applied Biosystems (Foster City, CA). *Pvu*I restriction enzyme was obtained from New England Biolabs (Beverly, MA).

Cell culture

All cell lines were grown as monolayers in tissue culture flasks or dishes in a 37°C, humidified incubator equilibrated with 5% CO_2. CHO-KI, CT60-HN, CT60-A1, and CT60-A13 cells were maintained in DMEM/F12 50:50 mix, whereas 25RA and CT60 cells were maintained in Ham's F12 medium. All media were supplemented with 2 mM glutamine, 1% Eagle's vitamins, 100 IU/ml penicillin, 100 μg/ml streptomycin, and the indicated serum. Growth medium for the CT60-HN, CT60-A1, and CT60-A13 cells was supplemented with 400 μg/ml geneticin. The FBS used in the experiments was heat inactivated by incubation at 56°C for 1 h. The lipoprotein-deficient serum was obtained by density gradient centrifugation of heat-inactivated calf serum at a density of >1.215 g/ml.

Cholesterol mass

Cholesterol mass was determined as described by Reagan, Hubbert, and Shelness (32). Briefly, lipids of cell homogenates were extracted by the method of Bligh and Dyer (33). A known amount of stigmasterol was included as an internal standard. Total and free cholesterol contents were determined in lipid extracts by gas liquid chromatography as described by Klansek et al. (34) using a Hewlett-Packard model 5890 gas chromatograph with autosampler. Separations were carried out at 240°C with an inlet and detector temperature of 270°C using a J&W Scientific 15-M megabore column coated with 50% phenylmethyl polysiloxane to a film thickness of 1.0 μm. Cholesteryl ester mass was calculated as the difference between total and free cholesterol and expressed as μg/mg cell protein, as determined by the method of Lowry et al. (35).

Cholesterol esterification

Cholesterol esterification was determined as described by St. Clair, Smith, and Wood (36). Briefly, [^{14}C]oleate was complexed to albumin such that the final specific activity was ~11,000 dpm/nmol. Fifty microliters of the complexed [^{14}C]oleate was added

to 5 ml of culture medium, resulting in 1 μCi/ml and a final oleate concentration of 0.17 mM. The cells were incubated for 24 h at 37°C before cellular lipids were extracted with ethanol, using tritiated-free cholesterol as an internal standard. The lipids were separated by thin-layer chromatography, and the free and esterified cholesterol bands were quantified by liquid scintillation counting and expressed as nmol/mg protein.

Sterol synthesis

Endogenous sterol synthesis was determined as described by Guertler and St. Clair (37). Cells were grown as described, and medium containing 5 μCi of [^{14}C]acetate that was previously diluted in sodium acetate was added for the final 2 h at a concentration of 1.67 μCi [^{14}C]acetate/ml and 0.75 mM acetate. After the 2 h incubation, cells were washed with BSS and lipids were extracted with isopropanol containing [^{3}H]free cholesterol as an internal standard. The lipids were saponified and subsequently separated by thin-layer chromatography in hexane-ether-acetic acid (146:50:4). Lipids were identified by exposure to iodine vapors, and the bands corresponding to free cholesterol were scraped and analyzed for radioactivity by liquid scintillation counting. Data were corrected for recovery of the [^{3}H]free cholesterol and expressed as dpm/mg cell protein/h (35).

Real-time quantitative RT-PCR

Total RNA was isolated with Trizol reagent according to the manufacturer's instructions. The cDNA was reverse transcribed from 1 μg of RNA using the Omniscript kit. Real-time quantitative RT-PCR was performed in an ABI PRISM 7000 thermocycler with the SYBR green Master Mix. The HMG-CoA reductase gene was amplified with the primers 5′-TGGCTACGATGTCTCCCTACA-3′ (forward) and 5′-CAACCCACACACCTGATGAA-3′ (reverse) at a concentration of 200 nM. The LDL receptor gene was amplified with the primers 5′-TGGTCATCCTCCTTGTCTTTG-3′ (forward) and 5′-TTCATCTTCTGTGGTCTTCTGG-3′ (reverse) at a concentration of 500 nM. The cyclophilin gene was amplified with the primers 5′-GTGGTCTTTGGGAAGGTGAA-3′ (forward) and 5′-TCGGAAATGGTGATCTTCTTG-3′ (reverse) at a concentration of 200 nM. PCR was performed using an initial 10 min denaturation at 95°C followed by 40 cycles of a two-step reaction (15 s at 95°C, then 1 min at 60°C). Data from each gene were normalized to cyclophilin with the Q-Gene software application (38).

Amplification of the SCAP gene

RNA from specified cell lines was subjected to reverse transcription. Each reverse transcription reaction contained 1 μg of RNA, 100 pmol of random primers, each deoxynucleoside triphosphate at a final concentration of 0.5 mM, 10 units of RNasin, 1× PCR buffer, and 4 units of Omniscript RT. The volume of each reaction was brought up to 20 μl with diethylpyrocarbonate-water. The reverse transcription was run as follows: 37°C for 1 h, followed by a 96°C incubation for 6 min. The cDNA was subjected to PCR with the indicated SCAP primers. Each PCR contained 1× Promega PCR Master Mix, 1 μM of each primer, and ~250 ng of cDNA template. The volume of each reaction was brought up to 25 μl with water. DNA amplification was carried out as follows: denaturation at 94°C for 3 min, followed by 25 cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 2 min. After 25 cycles, the reactions were incubated at 72°C for 10 min to increase the yield of amplification.

Sequencing

DNA sequencing was performed in the DNA Sequencing Laboratory of Wake Forest University School of Medicine on an Applied Biosystems model 3100 Genetic Analyzer. Samples undergo a cycle sequencing reaction that uses ABI Big Dye™ Terminator Chemistry. The fluorescently labeled products are separated from reaction reagents and analyzed by capillary electrophoresis. The DNA sequence is automatically analyzed by 3100 analysis software.

*Pvu*I digestion of PCR products amplified from the SCAP gene

DNA was amplified from CHO-KI, 25RA, CT60, CT60-HN, CT60-A1, and CT60-A13 cells using the PCR methods described above. The PCR products were purified on a 1% low-melting-temperature agarose gel and recovered with three sequential extractions of phenol, phenol-chloroform (1:1), and chloroform, followed by ethanol precipitation. Aliquots of the PCR products were incubated at 37°C for 1 h in the absence or presence of 13.5 units of *Pvu*I. The treated DNA samples were separated on a 1% agarose gel.

RESULTS

Expression of a functional NPC1 protein in an NPC1-deficient background influences cholesterol homeostasis

Throughout the studies described below, we used a number of CHO cell lines to elucidate the role of NPC1 in the regulation of cholesterol metabolism. CT60 and its parental cell line, 25RA, both express a mutant SCAP protein produced by a gain-of-function mutation that renders the cells resistant to the sterol-mediated suppression of cholesterol synthesis and lipoprotein uptake (4, 19). CT60 cells also express a truncated, nonfunctional NPC1 protein that results in lysosomal cholesterol accumulation (39). This cell line was complemented by Gu et al. (27) with a YAC containing the human NPC1 genetic interval to correct the mutation. CT60-A1 and CT60-A13 cells represent two neomycin-resistant sibling clones obtained through the introduction of a single YAC into the CT60 cells. CT60-HN cells serve as a control by expressing a YAC with hypoxanthine, aminopterin, and thymidine sensitivity and neomycin resistance but lacking the NPC1 interval.

Expression of the functional NPC1 protein in CT60-A1 and CT60-A13 cells reduced the lysosomal filipin staining indicative of accumulated cholesterol and increased cholesterol esterification (27). To determine what accounted for the decrease, cholesterol mass and esterification were analyzed in all of these cell lines. As a result of the SCAP mutation, cholesteryl ester concentration and esterification rates were increased in 25RA cells in the absence of LDL and were stimulated to an even greater extent in the presence of LDL compared with wild-type cells (**Fig. 1A**, **B**). Similar results were obtained for CT60 and CT60-HN cells, but the extent of cholesteryl ester accumulation and esterification was less than in 25RA cells as a result of the sequestration of cholesterol in the lysosomal compartment. Upon correction of the NPC1 defect in CT60-A1 and CT60-A13 cells, free cholesterol mass, esterified cholesterol mass, and cholesterol esterification rate were restored to wild-type levels. This was surprising given the ex-

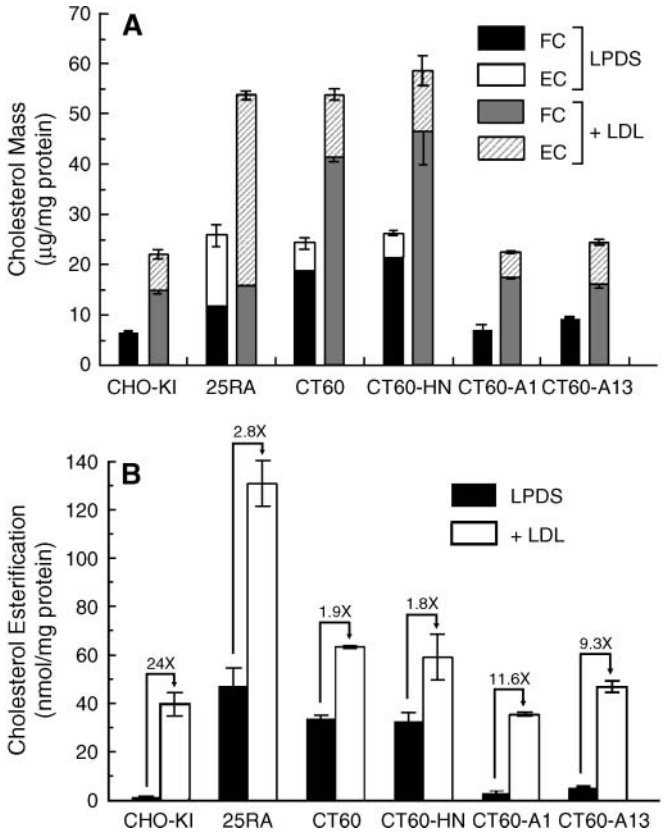

Fig. 1. Effect of Niemann-Pick type C1-yeast artificial chromosome (NPC1-YAC) complementation in the CT60 background on cholesterol mass and esterification. Cells were plated in medium containing 10% FBS. On the following day, the cells were washed and fresh medium containing either 10% lipoprotein-deficient serum (LPDS) or 10% LPDS + 50 μg/ml LDL was added. A: After a 48 h incubation, the cells were harvested and lipids were extracted from the cell homogenates by the method of Bligh and Dyer (33), and esterified (EC) and free (FC) cholesterol mass were quantified as described in Materials and Methods. B: After a 22 h incubation, 0.17 mM [^{14}C]oleic acid conjugated to albumin was added to the cells for an additional 2 h. The cells were washed and esterification was measured as described in Materials and Methods. Results represent means ± SD of triplicate samples. Fold changes in esterification rates upon LDL addition are displayed above the corresponding samples.

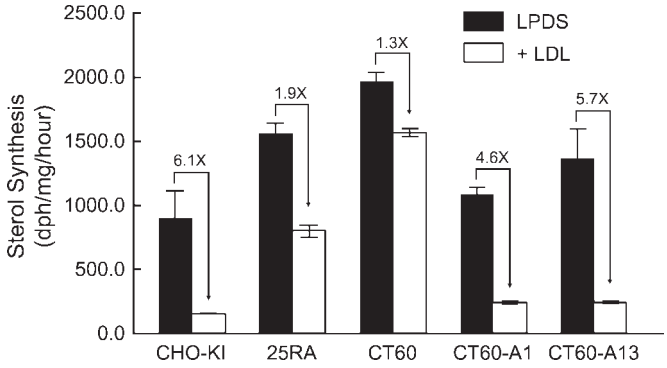

Fig. 2. Effect of NPC1-YAC complementation in the CT60 background on endogenous sterol synthesis. Cells were plated in 60 mm dishes in medium containing 10% FBS. On the following day, the cells were washed with BSS and incubated in the presence of medium containing either 10% LPDS or 10% LPDS + 75 μg/ml LDL for 24 h. Medium containing 5 μCi of [^{14}C]acetate that was previously diluted in sodium acetate was added for the final 2 h at a concentration of 1.67 μCi [^{14}C]acetate/ml and 0.75 mM acetate. Cells were harvested for the measurement of [^{14}C]acetate incorporation into sterols as described in Materials and Methods. Results represent means ± SD of triplicate samples. Fold changes in synthesis rates upon LDL addition are displayed above the corresponding samples.

pected presence of the gain-of-function SCAP mutation, which should increase esterified cholesterol concentrations and result in a phenotype that more closely resembles that of 25RA cells.

These results suggested that expression of the NPC1 protein in CT60-A1 and CT60-A13 cells overcomes the phenotypic changes in cholesterol metabolism associated with the SCAP mutation. To investigate this possibility further, endogenous sterol synthesis rates were measured (**Fig. 2**). Compared with wild-type cells, both 25RA and CT60 synthesis rates were increased significantly as a result of the SCAP mutation. Upon addition of LDL to the growth medium of 25RA and CT60 cells, synthesis was downregulated slightly as a result of posttranslational events (40), but to a much lesser extent than in wild-type cells, because of the absence of adequate transcriptional downregulation (1.9- and 1.3-fold compared with 6.1-fold, respectively). In CT60-A1 and CT60-A13 cells, basal levels of endogenous cholesterol synthesis and changes in the rate of synthesis after exposure to LDL were restored to wild-type levels (Fig. 2).

The transcription of a number of genes involved in cholesterol metabolism is regulated through the SREBP/SCAP pathway. Cell lines that possess a mutant SCAP protein (25RA and CT60) exhibit highly increased message levels of these specific genes, which consequently leads to increased cholesterol mass, esterification, and sterol synthesis as a result of the inability of cholesterol to downregulate the SREBP/SCAP pathway. To further assess the affect of NPC1 expression in CT60-A1 and CT60-A13 cells, message levels of both HMG-CoA reductase and the LDL receptor were measured by real-time quantitative RT-PCR. HMG-CoA reductase (**Fig. 3A**) and LDL receptor (Fig. 3B) message levels were increased significantly in 25RA, CT60, and CT60-HN cells as a result of the presence of the SCAP mutation. However, upon introduction of NPC1 in CT60-A1 and CT60-A13 cells, HMG-CoA reductase and LDL receptor message levels were reduced to wild-type levels. This reduction in message is consistent with the cholesterol metabolism experiments and supports the hypothesis that expression of a functional NPC1 protein in the CT60 background overcomes the altered regulation of sterol homeostasis induced by the SCAP mutation.

Identification and confirmation of the SCAP gain-of-function mutation

Restoration of cholesterol mass, esterification, and message levels of SREBP-regulated genes to normal in CT60-A1 and CT60-A13 cells suggests that the expression of NPC1 either affects the SREBP/SCAP pathway or inhibits the ex-

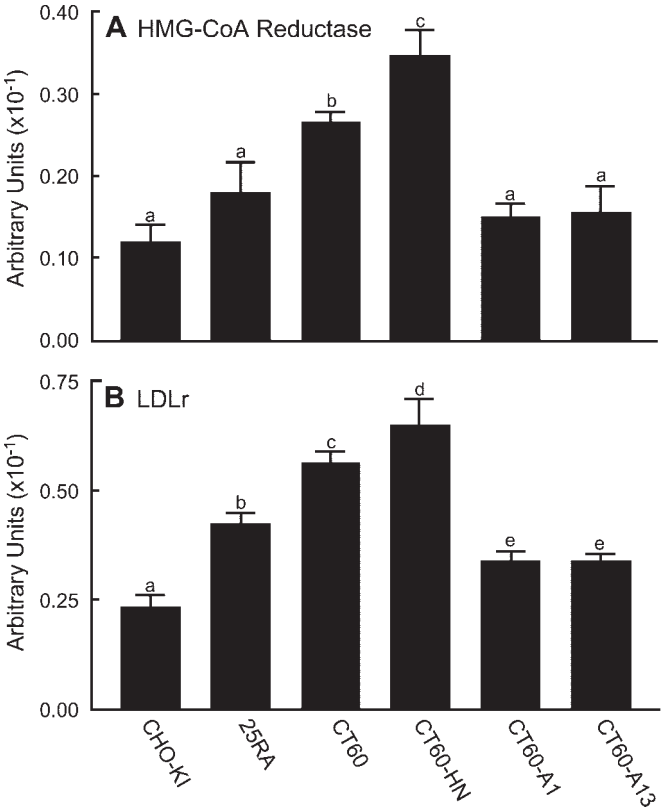

Fig. 3. Effect of NPC1-YAC complementation in the CT60 background on HMG-CoA reductase and LDL receptor message levels. Cells were plated in 100 mm dishes in medium containing 10% FBS. On the following day, the cells were washed with BSS and incubated in the presence of medium containing 10% LPDS for 48 h. RNA was harvested from the cells and message levels of HMG-CoA reductase (A) and the LDL receptor (LDLr; B) were determined by quantitative real-time RT-PCR as described in Materials and Methods. Results represent means ± SEM of triplicate determinations on each cDNA preparation. Significant differences are indicated by different letters as determined using the Bonferroni multiple comparison test ($P < 0.05$).

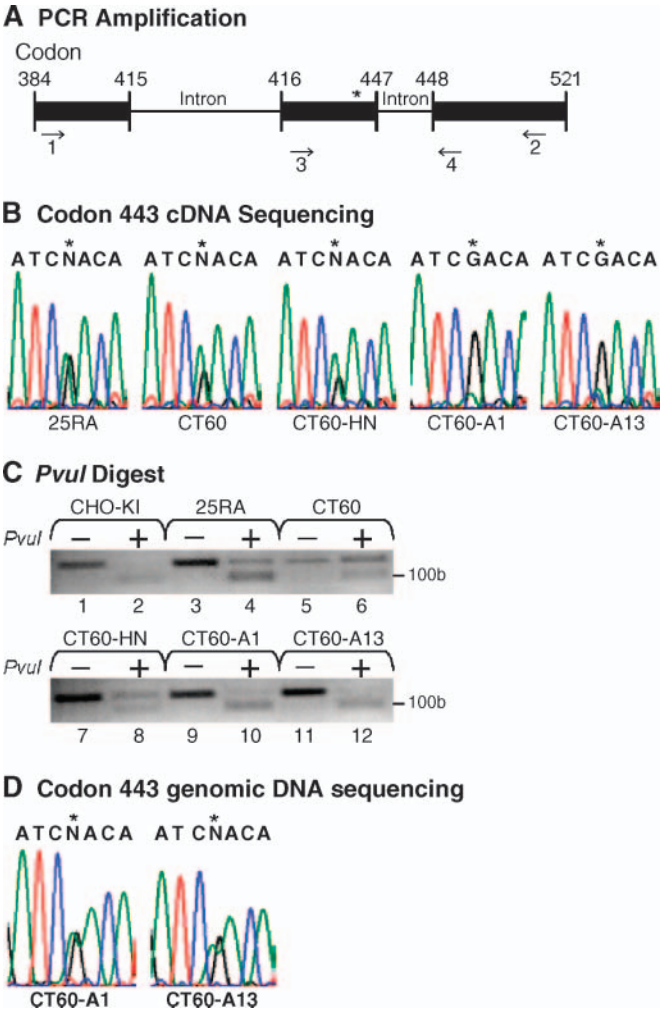

Fig. 4. Identification of the SCAP codon 443 mutation. A: The scheme represents the PCR amplification scheme for complementary and genomic DNA from the hamster SCAP gene. The asterisk denotes the location of the codon 443 mutation. The primers used for amplification in B and C are indicated below the scheme and are described in Table 1. B: RNA isolated from the indicated cell lines was reverse transcribed, and the cDNA product was amplified by PCR using primers 1 and 2. The PCR product was prepared for sequencing as described in Materials and Methods. Primer 1 was used for sequence analysis. Asterisks identify the position of the codon 443 mutation. C: Codon 443 of SCAP (nucleotides 1,152–1,528) was amplified by PCR in the indicated cell lines using primers 1 and 2 (Table 1). The PCR products were digested with *Pvu*I as described in Materials and Methods. D: Genomic DNA isolated from the indicated cell lines was amplified by PCR using primers 3 and 4. The PCR product was prepared for sequencing as described in Materials and Methods. Primer 4 was used for sequence analysis. Asterisks identify the position of the codon 443 mutation.

pression of the mutant SCAP protein. The specific defect in 25RA and CT60 cells is a transition mutation that converts an aspartic acid to asparagine as a result of a G-to-A substitution at codon 443 of the SCAP gene. This is a dominant mutation that is expressed in only one allele of the SCAP gene (27, 41). Because of this heterozygosity, sequencing of the gene presents both the wild-type and mutant alleles.

To determine whether CT60-A1 and CT60-A13 cells continue to express the mutant SCAP allele, RNA was isolated and used as a template for RT-PCR and subsequent sequencing of the SCAP gene using primers that flanked the area surrounding mutant codon 443. Amplification of cDNA templates from 25RA, CT60, CT60-HN, CT60-A1, and CT60-A13 cells was accomplished using primers 1 and 2 (**Fig. 4A**, **Table 1**). The PCR products were sequenced, and the results showed that 25RA, CT60, and CT60-HN cells, all of which possess the mutant SCAP gene, express both the wild-type and mutant alleles of SCAP, whereas CT60-A1 and CT60-A13 cells express only the wild-type allele (Fig. 4B).

The G-to-A mutation at SCAP codon 443 destroys a site for the restriction enzyme *Pvu*I. The presence of the mutation at codon 443, and the consequential inhibition of *Pvu*I digestion, results in a fragment of ~248 bp. Digestion of the wild-type sequence results in two fragments of ~125 bp each. To confirm the presence or absence of the SCAP mutation, the region incorporating the mutant codon was amplified by PCR using primers 1 and 2 (Fig. 4A, Table 1) and subsequently digested with *Pvu*I. Com-

TABLE 1. Primer sequences, positions, product sizes, and PCR annealing temperatures for mutational analysis of SCAP

Primer Pair	Sense (Primer Position)	Antisense (Primer Position)	Expected Size	Annealing Temperature
			bp	
1 and 2	5′-TTAAGCAGTGAGAGCTGGTCC-3′ (1,272–1,292)	5′-AGGAAGTAGATGACACGCAGTC-3′ (1,627–1,648)	358	53°C
3 and 4	5′-TCTGCCTCTTTGCTGTTGTGGG-3′ (1,369–1,390)	5′-GGGCAGACGCTTGTTCAGGT-3′ (1,471–1,490)	248	55°C
5 and 6	5′-TCATGGACATCGAGTGTCTG-3′ (2,446–2,465)	5′-AGCCTTTCCCAGTTCTCCTG-3′ (2,607–2,626)	181 (cDNA), 260 (genomic)	53°C

Primer sequences correspond to the published sequence of SCAP (GenBank U67060). The nucleotide position of each primer is indicated in parentheses. The PCR product size obtained for each primer pair is indicated in base pairs.

plete digestion of the CHO-KI-amplified product occurred (Fig. 4C, lane 2), signifying that these cells express only the wild-type allele of SCAP. *Pvu*I digestion of the amplified products from 25RA, CT60, and CT60-HN cells shows both digested and undigested products, attributable to the presence of the wild-type and mutant alleles (Fig. 4C, lanes 4, 6, and 8). However, *Pvu*I digestion of CT60-A1- and CT60-A13-amplified products results in complete digestion, indicating that the NPC1-complemented cell lines no longer express the mutant SCAP allele, thereby supporting the sequencing data (Fig. 4C, lanes 10 and 12).

Failure to express the mutant SCAP allele in CT60-A1 and CT60-A13 cells could result from two possible events: either the mutation has reverted to the wild-type sequence at codon 443, or the mutant allele has been silenced. Both of these occurrences would result in the apparent exclusive expression of the wild-type allele when the cDNA is sequenced. To distinguish between these possibilities, genomic DNA was isolated from CT60-A1 and CT60-A13 cells and used as a template for the amplification and sequencing of SCAP codon 443 using primers 3 and 4 (Fig. 4A, Table 1). Sequencing demonstrated that both alleles are present in CT60-A1 and CT60-A13 cells (Fig. 4D). This supports the notion that the mutant allele is still present in the gene but its expression is silenced, and it rules out the possibility that the mutation has reverted.

To unequivocally demonstrate that the SCAP mutation in CT60-A1 and CT60-A13 cells has been silenced rather than reverted, the presence or absence of an additional polymorphism at codon 816 of the SCAP gene was determined. This polymorphism consists of a G-to-A transition that changes a glycine to arginine on the mutant SCAP allele, but it does not affect the activity of SCAP (19). 25RA cells and its derivatives (CT60 and CT60-HN) are heterozygous for this polymorphism, expressing one wild-type and one mutant allele (41). Codon 816 of the SCAP gene was amplified from both cDNA and genomic DNA templates using primers 5 and 6 for both templates (**Fig. 5A**, Table 1). The cDNA from 25RA, CT60, and CT60-HN cells was amplified, whereas both the cDNA and the genomic DNA from CT60-A1 and CT60-A13 cells were amplified. Sequencing of the amplified cDNA products confirmed the expression of both alleles in 25RA, CT60, and CT60-HN cells, whereas only the wild-type allele was expressed in CT60-A1 and CT60-A13 cells (Fig. 5B). However, both the wild-type (G) and mutant (A) nucleotides are present in these cells, as indicated by sequencing of the genomic DNA (Fig. 5C). This is further evidence that the codon 443 mutation in NPC1-expressing CT60 cells is still present in the genome, although its expression is silenced.

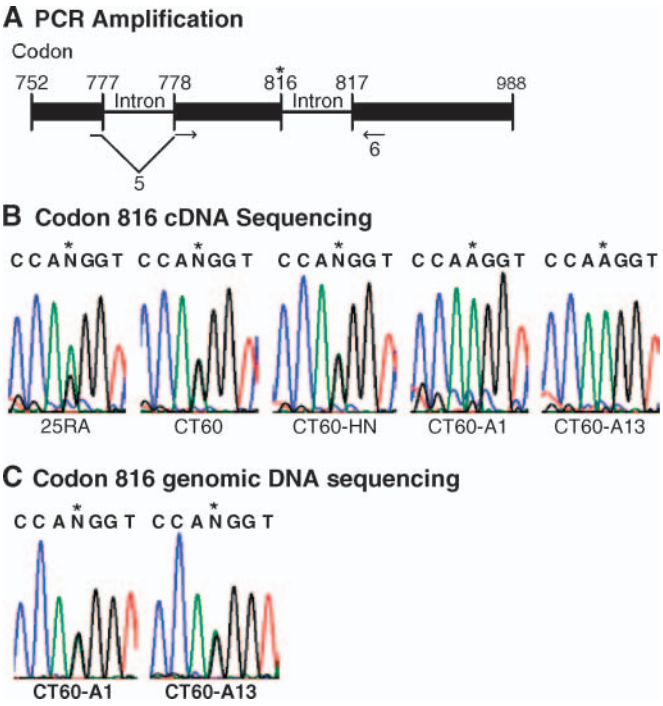

Fig. 5. Identification of the SCAP codon 816 silent polymorphism. A: The scheme represents the PCR amplification scheme for complementary and genomic DNA from the hamster SCAP gene. The asterisk denotes the location of the codon 816 polymorphism of the SCAP gene. The primers used for amplification in B and C are indicated below the scheme and are defined in Table 1. B: RNA isolated from the indicated cell lines was reverse transcribed, and the cDNA product was amplified by PCR using primers 1 and 2. The PCR product was prepared for sequencing as described in Materials and Methods. Primer 1 was used for sequence analysis. Asterisks identify the position of the codon 816 mutation. C: Genomic DNA isolated from the indicated cell lines was amplified by PCR using primers 5 and 6. The PCR product was prepared for sequencing as described in Materials and Methods. Primer 5 was used for sequence analysis. Asterisks identify the position of the codon 816 polymorphism.

Specificity of SCAP mutation silencing

The mechanism by which NPC1 expression silences the SCAP mutation in the CT60 background is unknown. To determine whether it results from a specific function of the NPC1 protein, we sequenced the codon 443 SCAP mutation in a CT60-NPC1 cell line. This line stably expresses the mouse NPC1 cDNA tagged with the EYFP fluorescent protein (25). If silencing of the SCAP mutation is a result of NPC1 expression, then these cells, like CT60-A1 and CT60-A13 cells, should express only the wild-type SCAP allele. However, the sequence data indicated that the mutant SCAP allele is still present and expressed in CT60-NPC1 cells (**Fig. 6A**); therefore, a specific effect of NPC1 expression can be ruled out.

Silencing of the gain-of-function SCAP mutation could also represent a selective advantage induced by increased cellular cholesterol levels. The CHO mutant cell line AC29 was used to test this hypothesis. AC29 cells also possess a gain-of-function SCAP mutation in addition to a defect in the ACAT protein, which prevents the esterification of free cholesterol within the ER (21). This cell line exhibits increased rates of endogenous sterol synthesis and an inability to esterify cholesterol; therefore, the high concentrations of free cholesterol that fail to be esterified for storage may have toxic effects on the cell. Normal regulation of cholesterol metabolism resulting from silencing of the SCAP mutation would reduce the possibility that potentially harmful concentrations of cholesterol would accumulate. However, sequencing of SCAP codon 443 in AC29 cells revealed that these cells continue to express the mutant SCAP gene (Fig. 6B). This does not support the theory that silencing of the SCAP mutation confers a selective advantage for specific cell lines.

DISCUSSION

It was shown previously that CHO-KI cells and several CHO-KI mutants (25RA, CT60, and CT60-HN) derived by chemical mutagenesis express both alleles of the SCAP gene. The data presented here confirm this observation and demonstrate that, unlike many CHO genes, the SCAP gene is not functionally haploid (19, 41). Despite the expression of one mutant and one normal SCAP allele, the gain-of-function phenotype is still observed as a result of the dominance of this mutation, which conveys resistance to the sterol-mediated suppression of many genes involved in the maintenance of intracellular cholesterol homeostasis (42). Sequence analysis confirmed that the mutation is still present in the genome of the NPC1-complemented cell lines (CT60-A1 and CT60-A13) but is no longer expressed, as it is in CT60 cells. Consequently, increased expression of proteins that contribute to increased cholesterol uptake and synthesis is downregulated in CT60-A1 and CT60-A13 cells.

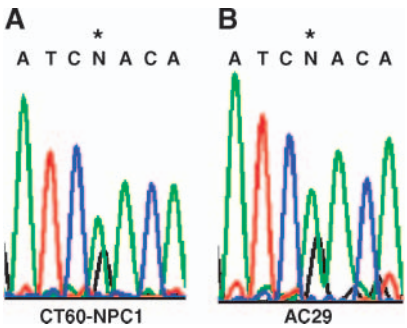

Fig. 6. Specificity of SCAP mutation silencing. RNA isolated from the CT60-NPC1 stable cell line (A) and AC29 cells (B) was reverse transcribed, and the cDNA product was amplified by PCR using primers 1 and 2. The PCR product was prepared for sequencing as described in Materials and Methods. Primer 1 was used for sequence analysis. Asterisks identify the position of the codon 443 mutation.

The CT60-A1 and CT60-A13 cell lines played a critical role in the identification of the NPC1 gene (27). The initial characterization of these cell lines demonstrated that correction of the NPC1 defect by expression of the NPC1 genetic interval significantly increased the esterification of LDL-cholesterol compared with CT60 esterification levels (27). However, this increase was expressed as the overall change in cholesterol oleate synthesis induced by LDL addition, rather than as the absolute amount of cholesteryl ester synthesized before and after LDL supplementation. Presentation of the data in this manner accurately reflected changes that resulted from the expression of a functional NPC1; however, the decrease in cholesteryl ester mass, attributable to silencing of the SCAP mutation, was not evident. Based on our findings, we presume that the esterification rates in CT60-A1 and CT60-A13 cells, described by Gu et al. (27), were comparable with wild-type levels rather than with the increased rates observed in 25RA cells.

The mechanism by which the expression of NPC1-YAC silences the SCAP mutation is unknown. Failure of NPC1 cDNA stable expression in CT60 cells to induce silencing of the mutant SCAP allele suggests that it is not a function of the NPC1 protein itself (Fig. 6A). In fact, the NPC1 gene spans only ~100 kb of the 590 kb YAC, leaving the potential for 10–20 additional genes to be expressed, which could alter the expression of SCAP. Indeed, although NPC1 was the most abundant product, two other genes were expressed: the Soares ovary tumor cDNA and an additional product that shared no homology with any known mammalian genes or expressed sequence tags (43). In addition, a number of endogenous hamster genes were thought to be upregulated after YAC transduction. These included the cell proliferation gene Ki-76, Y-b3 glutathione *S*-transferase, and the cholecystokinin receptor (43). The ability of YAC expression to upregulate endogenous genes suggests that its expression could also silence certain genes, thus accounting for the silencing of the mutant SCAP allele. Alternatively, silencing of the SCAP gene could be induced by methylation of the promoter region, a common cause of hemizygosity demonstrated in genes such as the tumor suppressors Rb and p16 (44, 45), as well as thymidine kinase (46). Methylated genes are known to be stably inherited in their active and inactive forms, which would

account for the perpetuation of a cell line that expressed only the normal SCAP allele (47). Although this is a credible possibility, treatment of CT60-A1 and CT60-A13 cells with 5-azacytidine, a methylation inhibitor, failed to restore the expression of the mutant SCAP allele, suggesting that silencing is not induced through this mechanism (data not shown). It is also possible that silencing of the mutant SCAP allele occurred during the clonal selection of CT60-A1 and CT60-A13 cells. Stable integration of the NPC1-expressing YAC could occur at a higher frequency in cells that had silenced the mutant allele. Alternatively, silencing could have occurred as a secondary alteration at the epigenetic level during the extended isolation process.

The occurrence of selective allele silencing in both characterized cell lines (CT60-A1 and CT60-A13) suggests that this was not a random event; rather, the cells were induced to make this change. Theoretically, silencing of the SCAP mutation could signify the induction of a selective advantage in cells that have abnormally high concentrations of free cholesterol. CT60-NPC1 and AC29 cells both represent cell lines that exhibit an overproduction of endogenous cholesterol, coupled with the inability to sequester the cholesterol within lysosomes or convert it to cholesteryl ester, respectively. Although CT60 cells have been complemented with a functional NPC1 protein, transport of an excessive amount of cholesterol to the plasma membrane may disrupt the lipid balance. Similarly, AC29 cells have lost their ability to effectively store free cholesterol in esterified form. Therefore, silencing of the SCAP mutation and the subsequent decrease in endogenously synthesized cholesterol could provide these cells with an advantage over cells still expressing the mutant gene. However, such a selective advantage apparently does not exist, because both of these cell lines continue to express the mutant SCAP allele.

These data provide a unique example of gene silencing through the stable expression of a YAC. Because all of the genes expressed by the YAC have not been identified, we are unable to determine whether the expression of a specific gene has resulted in silencing of the mutant SCAP allele or whether alterations to endogenous gene expression are the cause. Regardless of the mechanism, these data reveal the possibility of secondary changes that may accompany YAC expression and highlight the necessity of cautious interpretation regarding phenotypic changes that extend beyond those induced by the particular gene of interest. Furthermore, they demonstrate the complementary roles of NPC1 and the SREBP/SCAP pathway in the maintenance of cholesterol homeostasis. Although the data do not eliminate the possibility of an interaction between NPC1 and the SREBP/SCAP pathway, further study using other systems will be required to definitively establish such a relationship.

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