Complementation of Mutation in Acyl-CoA:Cholesterol Acyltransferase (ACAT) Fails to Restore Sterol Regulation in ACAT-defective Sterol-resistant Hamster Cells*

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A previously described mutant line of Chinese hamster ovary cells, designated SRD-4, fails to synthesize cholesteryl esters, owing to a deficiency in the activity of acyl-CoA:cholesterol acyltransferase (ACAT). These cells also fail to suppress low density lipoprotein receptors or cholesterol synthesizing enzymes in the presence of 25-hydroxycholesterol. In the current studies we show that SRD-4 cells have three defects: 1) a point mutation in one allele at the ACAT locus that changes codon 265 from Ser to Leu, resulting in an inactive enzyme; 2) a silent allele at the other ACAT locus that does not produce detectable mRNA; and 3) a mutation, as yet undefined, that abolishes the ability of 25-hydroxycholesterol to inhibit the cleavage of both sterol regulatory element binding proteins (SREBP-1 and SREBP-2). Correction of the ACAT deficiency by transfection of a wild-type cDNA failed to restore inhibition of SREBP cleavage by 25-hydroxycholesterol, indicating that the ACAT deficiency and the sterol regulatory defect are caused by independent mutations. These data provide further insight into the interplay between ACAT activation and inhibition of SREBP cleavage by 25-hydroxycholesterol, and they indicate that these two processes can be disrupted independently by mutation.

A major step in the molecular understanding of ACAT came from the cDNA cloning of the human enzyme by Chang et al. (4). These workers used a clever selection technique to obtain a line of Chinese hamster ovary (CHO) cells, designated AC29, that is deficient in ACAT activity and therefore fails to accumulate cholesteryl ester droplets when incubated with LDL or 25-hydroxycholesterol (5). They were able to reverse this defect by transfection of human genomic DNA into the cells (6). This eventually led to the isolation of a human cDNA encoding ACAT (4). The protein turned out to be hydrophobic with areas of homology to other acyltransferases. Expression of this protein in insect Sf9 cells, which do not themselves express ACAT, yielded a high level of ACAT activity, confirming that the cDNA encoded the catalytic component (7).

The AC29 cell line that Chang et al. (4) used as a recipient in the cloning studies has two defects, 1) it lacks the ACAT enzyme (8); and 2) it is resistant to feedback repression of cholesterol biosynthesis and LDL receptor activity by 25-hydroxycholesterol (5, 6). These two defects arose independently. The cells were first selected for 25-hydroxycholesterol resistance, and they were subsequently selected for ACAT deficiency (5). The molecular nature of the mutation that abolishes ACAT expression and the nature of the defect that leads to a failure of 25-hydroxycholesterol regulation in AC29 cells is unknown.

Our laboratory also isolated a mutant CHO cell line, designated SRD-4 cells, with the same two defects, 1) lack of ACAT activity; and 2) resistance to 25-hydroxycholesterol-mediated feedback regulation (9). Although the SRD-4 cells emerged from a single step selection, we concluded that they also had two independent mutations because treatment of wild-type cells with an ACAT inhibitor blocked cholesteryl esterification, but it did not reproduce the 25-hydroxycholesterol resistance phenotype (9).

25-Hydroxycholesterol represses cholesterol biosynthesis and LDL receptor activity at the transcriptional level by regulating a pair of proteins designated sterol regulatory element binding protein-1 and -2 (SREBP-1 and -2) (Refs. 10–12). These proteins are synthesized as integral components of the membranes of the ER and nuclear envelope (12–14). In sterol-depleted cells, a protease clips each protein to release an NH2-terminal fragment of ~500 amino acids that contains a basic-helix-loop-helix-leucine zipper motif and a transcription activating domain (12–14). This fragment, designated the "mature" form of SREBP, enters the nucleus and binds to sterol regulatory elements in the enhancer regions of the genes encoding 3-hydroxy-3-methylglutaryl-CoA synthase, an early enzyme of cholesterol biosynthesis, the LDL receptor (10–13), and other enzymes of sterol biosynthesis (15). Binding leads to transcriptional activation, which allows cells to increase their rate of de novo cholesterol synthesis and uptake of cholesterol from LDL through LDL receptors (10–13). SREBP-1 also activates transcription of the genes encoding acyl-CoA carboxyl-
ASE and fatty acid synthetase, two enzymes of fatty acid biosynthesis (16, 17). When LDL-derived cholesterol or 25-hydroxycholesterol overaccumulates in cells, the proteolysis of SREBP2 is reduced, the proteins remain membrane-bound, and transcription of the target genes declines (10–14).

We previously described three mutant lines of CHO cells, designated SRD-1, -2, and -3 cells, that constitutively transcribe the genes encoding enzymes of cholesterol biosynthesis and the LDL receptor (18–21). These cells show virtually no suppression when 25-hydroxycholesterol is added. In contrast to the SRD-4 cells, the SRD-1, -2, and -3 cells have normal ACAT activity that is stimulated normally by LDL or 25-hydroxycholesterol (20, 21).

The SRD-1, -2, and -3 cells have each undergone genomic recombinations that yield rearranged mRNAs encoding a truncated form of SREBP-2 that terminates before the membrane attachment domain (18, 19). Because the truncated SREBP-2 is never attached to the membrane, it does not require proteolysis, and, therefore, it is always active; 25-hydroxycholesterol never enters the membrane, it does not require proteolysis, and, therefore, it is always active; 25-hydroxycholesterol cannot suppress its activity.

The SRD-4 cells, like the SRD-1, -2 and -3 cells, show non-regulated expression of the enzymes of cholesterol biosynthesis and the LDL receptor (9). We do not know whether the defect in these cells is caused by the production of a truncated form of SREBP. Moreover, we do not know the reason why these cells, in contrast to the SRD-1, -2 and -3 cells, lack ACAT activity.

In the current experiments we have used the human cDNA clone of Chang et al. (4) to isolate a cDNA encoding wild-type hamster ACAT. We have found that the SRD-4 cells produce a form of ACAT with a point mutation that changes a conserved amino acid and abolishes activity of the enzyme. We also show that the SRD-4 cells do not produce a truncated form of SREBP; rather, they continue to process the wild-type hamster ACAT.

Finally, we show that the ACAT defect by transfection with wild-type ACAT fails to correct the regulatory response to 25-hydroxycholesterol in SRD-4 cells, confirming that these two abnormalities arose from independent mutations.

EXPERIMENTAL PROCEDURES

Materials and Methods—We obtained HSV-Tag™monomonal antibo-
dies from Novagen. [14C]Cleavage (55 mCi/μmol) and [35S]Cleavage-CoA (53 mCi/μmol) were purchased from DuPont-NEN. Standard molecular biology techniques were used (22). DNA sequencing was performed with the dyeodeoxy chain termination method on an Applied Biosystems model 373A DNA sequencer. Probes were radiolabeled with [32P]dCTP by the random primer method using the Prime-It™II kit (Stratagene). Plasmids were prepared with Qiagen (midi or maxi prep) or Promega (mini prep) plasmid kits. CHO-7 and SRD-4 cells were described previously (9). Expression plasmids for HSV-tagged human SREBP-1 and SREBP-2, designated pTK-HSV-BP1 and pTK-HSV-BP2, respectively, were described previously (23, 24). Human LDL (d 1.019–1.063 g/ml) and newborn calf lipoprotein-deficient serum (d > 1.215 g/ml) were prepared as described previously (24).

cDNA Cloning of Hamster Wild-type ACAT—A 1.52-kb cDNA library from hamster SRD-2 cells (13) was screened with a [32P]-labeled 1.8-kb BamHI/NcoI cDNA fragment encoding human ACAT (4) at 42°C overnight in 35% (v/v) formamide, 5× SSPE, 5× Denhardt’s solution, 100 μg/ml salmon sperm DNA, and 0.5% SDS. The blots were washed twice with 2× SSC for 30 min at 50°C and once with 0.5× SSC at 50°C. Among 5× 108 plaques screened, four positive clones were identified. The clone with the longest insert, 3.0 kb, was subcloned into the pBR322-CMV vector (Stratagene), sequenced on both strands, and subcloned into the CMV-driven, neo-containing expression vector pRcCMV75B (13). The resulting plasmid is designated pCMV-ACAT.

Blot Hybridization of RNA and DNA—Poly(A+)-RNA was isolated with oligo(G) cellulose (Stratagene) and loaded onto a 1.5% agarose gel in 40 mM MOPS. After electrophoresis at 50 mA for 4 hours at room temperature, the RNAs were transferred overnight onto Hybond-N membranes (Amersham Corp.) in 20× SSC, cross-linked with UV light, prehybridized for 30 min, and hybridized for 2 hours at 65°C in Rapid-hyb Buffer (Amersham Corp.) with a [32P]-labeled 1.6-kb PCR fragment corresponding to the coding region of the hamster ACAT cDNA (1× 106 cpm/ml). The blot was washed at room temperature with 2× SSC, 0.1% (w/v) SDS for 20 min, and 0.5× SSC/0.1% SDS twice at 65°C for 20 min, followed by autoradiography.

SRD-4 and CHO cells were digested with restriction enzymes, subjected to electrophoresis on a 0.7% agarose gel. The DNAs were transferred to Hybond-N membranes, cross-linked with UV light, and blotted with 1× 106 cpm/ml of the [32P]-labeled 1.6-kb hamster ACAT probe (see above) in Rapid-hyb Buffer. The blot was then washed twice with 0.2× SSC at 42°C followed by autoradiography.

PCR, SSCP Analysis, and Localization of Mutation in ACAT—To detect mutations in the hamster ACAT mRNA in SRD-4 cells, poly(A+) RNA from SRD-4 and CHO-7 cells was reverse-transcribed with the Stratascript™ reverse transcription-PCR Kit (Stratagene). The resulting RNA/DNA hybrids from each cell line were used as templates with [32P]dCTP in eight separate PCR reactions that were designed to cover the entire 1.6-kb coding region of the hamster ACAT. The resulting eight [32P]-labeled PCR products spanning the ACAT cDNA in both CHO-7 and SRD-4 cells were then subjected to SSCP analysis as described (25). One of the PCR products in the region of codon 265 gave an abnormal pattern in the SSCP analysis. To localize the mutation in this region, we sequenced SRD-4 mRNA by PCR with primers from the 3′ untranslated regions of the ACAT mRNA. A specific primer corresponding to codons 201–208 was used to sequence this region of the PCR product.

Site-directed Mutagenesis—In vitro mutagenesis was done with a Mutagen-m gene M13 In Vitro Mutagenesis Kit (Bio-Rad). A point mutation (Ser→Leu) at amino acid residue 265 in the wild-type ACAT cDNA was produced using a 27-base pair mutagenic oligonucleotide, 5′-CTCTCT-GACAAACAGTGACGCTTCAT-3′. The replication form DNA of the mutant cDNA (cloned in M13mp 19) was digested with Sall and NotI, and the insert was subcloned into pRcCMV75B (13). This plasmid was designated pCMV-ACAT(S265L). The mutation was confirmed by DNA sequencing.

Transient Transfection of 293 Cells—Monolayers of human embryonic kidney 293 cells were set up on day 0 at 4 × 105 cells/60-mm dish in Dulbecco’s modified Eagle’s medium with low glucose supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal calf serum. After incubation for 48 h at 37°C in a 5% CO2 incubator, the cells were transfected with the wild-type or mutant pCMV-ACAT plasmid using the MBS Transfection Kit (Stratagene) according to the manufacturer’s instructions. After incubation for 3 h at 35°C in a 3% CO2 incubator, the cells were washed once with phosphate-buffered saline, re-fed with fresh medium containing 10% new-born calf lipoprotein-deficient serum (medium A), returned to a 37°C/5% CO2 incubator, and used for experiments 16 h later.

Stable Transfection of SRD-4 Cells—Cells were seeded on day 0 at a density of 5 × 106 cells/60-mm dish in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% newborn calf lipoprotein-deficient serum and transfected on day 1 with 5 μg of hamster wild-type pCMV-ACAT using the MBS Transfection Kit as described above. Stable transformants from two dishes were selected by growth in medium supplemented with 700 μg/ml G418. G418-resistant colonies were picked and screened for incorporation of [14C]leucine into choleryl [35S]leucine by intact monolayers (see below). Positive colonies were cloned by dilution plating and rescreened. Four clones that stably expressed ACAT were isolated, and one of these, designated Tr-10–9, was used for experiments.

Immunoblot Analysis—Protein concentrations were measured with a BCA kit (Pierce). After SDS electrophoresis in a 12% gel, the proteins were transferred to Hybond-C extra transfer membrane (Amersham Corp.). Immunoblot analysis was carried out with Enhanced Chemiluminescence Western blotting Detection Kit (Amersham Corp.) according to the manufacturer’s instructions with the following conditions (14, 23). Filters were exposed to Reflection™ NEF film (DuPont).

Immunodetection of hamster ACAT was carried out with 10 μg/ml rabbit polyclonal antibody (Igs fraction) directed against a glutathione S-transferase fusion protein containing amino acids 1–140 of hamster ACAT. Immunodetection of endogenous hamster SREBP-1 and -2 was carried out with 3 μg/ml monoclonal IgGs-ZA4 (13) and 5 μg/ml monoclonal IgGs-ZD4 (19). Immunodetection of transfected HSV-tagged human SREBP-1 and -2 was carried out with 0.5 μg/ml of HSV-Tag™ monoclonal antibody (14, 23).
ACAT Assays—The rate of incorporation of \(^{14}C\)oleate into cholesteryl \(^{14}C\)oleate and \(^{14}C\)triglycerides by intact cell monolayers was measured as described previously (24).

ACAT activity in membranes from cell extracts was assayed by measuring the rate of conversion of \(^{1-14}C\)oleoyl-CoA to cholesteryl \(^{14}C\)oleate as described previously (3, 9) with several modifications. The 2,000 \(^{3}g\) cell pellets were resuspended in buffer A (50 mM potassium phosphate and 2 mM dithiothreitol at pH 7.4), homogenized by 10 passages through a 25-gauge needle, and subjected to centrifugation at 105 \(^{3}g\) for 1 h at 4°C to yield a total cell membrane fraction. This fraction was suspended in buffer A. Membrane fractions (25–100 \(mg\) of protein) were incubated in a final volume of 0.2 ml for 20 min at 37°C in buffer A containing 5 mg/ml bovine serum albumin, followed by incubation for 20 min at 37°C with 10 \(mg/ml\) cholesterol (added in 2 \(ml\) of ethanol). \(^{14}C\)Oleoyl-CoA (20 dpm/pmol) was then added to a final concentration of 75 \(mM\), and the incubations were continued for 1 h at 37°C. Reactions were terminated by addition of chloroform/methanol (1:1, v/v) continued 20,000 dpm \[^{3}H\]cholesteryl oleate as a recovery control. Neutral lipids were extracted and resolved by thin layer chromatography as described previously (24).

RESULTS

We isolated a cDNA encoding hamster ACAT by probing a Lgt22A cDNA library prepared from SRD-2 cells (13), a line of CHO cells that has high ACAT activity, owing to overproduction of cholesterol as a result of a dominant positive mutation in SREBP-2 (19). Fig. 1 shows that the amino acid sequence of hamster ACAT, as deduced from the cDNA sequence, is 88 and 92% identical to the human and mouse homologues, respectively. The hamster and mouse proteins are each slightly shorter than the human sequence (546 and 540 amino acids, respectively, versus 550 amino acids), owing to deletions of blocks of four or six residues.

Fig. 2 shows a Northern blot showing that the wild-type CHO-7 cells and the SRD-4 cells each produced a single ACAT mRNA of \(3.3kb\) (Fig. 2). The relative abundance was similar in the two cell lines, but a 2-fold difference could not have been detected by this technique.

To search for a point mutation in the ACAT mRNA in SRD-4 cells, we made a cDNA copy of the mRNA with reverse transcriptase, and then we used oligonucleotide primers with \[^{32}P\]dCTP to amplify by PCR eight overlapping segments that covered the entire 1.6-kb coding region. The PCR products were denatured and subjected to electrophoresis in nondenaturing gels so as to detect single-strand conformational polymorphisms (SSCPs) (25). Seven of the amplified fragments were identical for the cDNAs from CHO-7 and SRD-4 cells (data not shown). The eighth fragment consistently showed a slight mobility difference between the two cell strains (Fig. 3). After denaturation, both strands of the SRD-4 cDNA (lane 4) were displaced relative to the CHO-7 strands (lane 3), and there was no evidence of any wild-type strands.

To identify the putative point mutation in the SRD-4 ACAT, the entire coding region of the mRNA was PCR-amplified, and the DNA sequence in the region of the abnormal SSCP was determined. This sequence showed a single nucleotide abnormality (C \(\rightarrow\) T) at the central position of codon 265, changing the amino acid from Ser to Leu. The sequence of the PCR product showed only a T and no C at this position, indicating that the SRD-4 cells produced only the abnormal species of mRNA and none of the normal transcript (data from automated
residues 218–226 and 269–275 in the wild-type hamster ACAT cDNA.

ACAT from all three known species (lanes 1 and 2) was reverse-transcribed into mRNA. Southern blots of genomic DNA, probed with the full-length cDNA, failed to reveal an abnormal fragment in the SRD-4 gene after digestion with several restriction enzymes, indicating that the silent allele does not have a gross deletion or rearrangement (data not shown).

We conclude that the SRD-4 cells are compound heterozygotes. One allele contains a C → T substitution at codon 265 and produces a mutant ACAT mRNA. The second allele contains a wild-type sequence at codon 265 and is both unstable and inactive.

To determine whether the S265L mutant has enzymatic activity, we transfected 293 cells with 5 μg of mutant ACAT plasmid and 1 μg of wild-type plasmid so as to obtain approximately the same amount of protein (see immunoblot in Fig. 5). The cells were lysed, a membrane pellet was prepared, and ACAT activity was measured with varying amounts of substrates. Membranes from 293 cells transfected with an empty vector had endogenous ACAT activity (open triangles, Fig. 5). Transfection with 1 μg of wild-type ACAT cDNA led to a several fold increase in enzyme activity (closed symbols). In contrast, transfection with 5 μg of the mutant cDNA produced no detectable increase over the endogenous activity (open triangles). Similar results were obtained in four separate experiments. We conclude that the S265L mutant of hamster ACAT is both unstable and inactive.

Next we conducted studies to determine whether the ACAT defect in SRD-4 cells causes the defect in sterol-mediated inhibition of the cleavage of SREBP and whether this latter defect would be corrected by correction of the ACAT defect. For this purpose, we transfected the wild-type ACAT cDNA into SRD-4 cells under control of the CMV promoter and isolated a permanent line of SRD-4 cells, designated Tr.10–9, that expresses the wild-type enzyme. Table II shows that monolayers of CHO-7 cells incorporated [3H]oleate into cholesterol and that the reaction was stimulated by addition of sterols in the form of 25-hydroxycholesterol plus LDL. The SRD-4 cells syn-

TABLE I

Clonal analysis of PCR products amplified from ACAT mRNA and genomic DNA from SRD-4 cells

| Template for PCR | DNA sequence at codon 265 |
|-----------------|---------------------------|
| Wild-type       | Mutant                     |
| TCG (Ser)       | TTG (Leu)                 |
| Poly(A-) RNA    | 0                          | 9                          |
| Genomic DNA     | 2                          | 2                          |

Fig. 2. Northern gel analysis of ACAT mRNA in CHO-7 and SRD-4 cells. A 32P-labeled 1.6-kb hamster ACAT probe was hybridized to poly(A+) RNA (5 μg/lane) from the indicated cell line as described under "Experimental Procedures." The filter was exposed to Reflection™ NEF film (DuPont) for 12 h at -70 °C. RNA size markers (Life Technologies, Inc.) are shown at the left.

Fig. 3. SSCP analysis of ACAT cDNA from CHO and SRD-4 cells. The reverse transcription products corresponding to the ACAT cDNA from CHO-7 (lanes 1 and 3) and SRD-4 (lanes 2 and 4) cells were incubated with oligonucleotide primers corresponding to amino acid GCTTTTTCTTCTTAGTCTT-3′. PCR was carried out in the presence of [3H]dCTP for 30 cycles at 96 °C for 1 min/66 °C for 2.5 min. Aliquots of the PCR products were denatured at 95 °C for 5 min in formamide and applied to a 6% glycerol polyacrylamide gel (lanes 3 and 4) adjacent to aliquots of the non-denatured samples (lanes 1 and 2). The gel was subjected to electrophoresis at 300 V for 16 h at room temperature, dried, and exposed to Reflection™ NEF film for 12 h at room temperature.

The data from the SSCP analysis (Fig. 3), the direct sequencing of the PCR product, and the subsequent analysis of cDNA clones (Table I) all indicated that the SRD-4 cells produced only the mutant form of the mRNA. To analyze the situation at the genomic level, we PCR-amplified the region of the gene containing codon 265 and cloned the products into a plasmid vector. Four independent colonies were isolated, and the plasmids were subjected to sequencing. Two of these plasmids showed the wild-type sequence at codon 265 (TCG), and the other two showed the mutant sequence (TTG) (Table I). We conclude that the SRD-4 cells are compound heterozygotes. One allele contains a C → T substitution at codon 265 and produces a mutant ACAT mRNA. The second allele contains a wild-type sequence at codon 265, but it is a silent allele that is not transcribed into mRNA. Southern blots of genomic DNA, surrounding codon 265 was sequenced. Genomic DNA from SRD-4 cells when the cells were incubated either in the absence or presence of sterols (lanes 1 and 2, respectively). When the 293 cells were transfected with a cDNA encoding wild-type hamster ACAT under control of the CMV promoter, a band in the correct size range was detected, and this was not altered by the addition of sterols (lanes 5 and 6). Transfection of the mutant cDNA produced a much lighter band (lanes 3 and 4). This experiment was replicated several times, and we interpreted it to indicate that the S265L mutation renders the protein unstable.

The cells were lysed, a membrane pellet was prepared, and ACAT activity was measured with varying amounts of substrates. Membranes from 293 cells transfected with an empty vector had endogenous ACAT activity (open triangles, Fig. 5). Transfection with 1 μg of wild-type ACAT cDNA led to a several fold increase in enzyme activity (closed symbols). In contrast, transfection with 5 μg of the mutant cDNA produced no detectable increase over the endogenous activity (open triangles). Similar results were obtained in four separate experiments. We conclude that the S265L mutant of hamster ACAT is both unstable and inactive.

We next conducted studies to determine whether the ACAT defect in SRD-4 cells causes the defect in sterol-mediated inhibition of the cleavage of SREBPs and whether this latter defect would be corrected by correction of the ACAT defect. For this purpose, we transfected the wild-type ACAT cDNA into SRD-4 cells under control of the CMV promoter and isolated a permanent line of SRD-4 cells, designated Tr.10–9, that expresses the wild-type enzyme. Table II shows that monolayers of CHO-7 cells incorporated [3H]oleate into cholesterol and that the reaction was stimulated by addition of sterols in the form of 25-hydroxycholesterol plus LDL. The SRD-4 cells syn-

FIG. 3. SSCP analysis of ACAT cDNA from CHO and SRD-4 cells. The reverse transcription products corresponding to the ACAT cDNA from CHO-7 (lanes 1 and 3) and SRD-4 (lanes 2 and 4) cells were incubated with oligonucleotide primers corresponding to amino acid residues 218–226 and 269–275 in the wild-type hamster ACAT cDNA. The sequences of these two primers, respectively, are 5′-GCCACG-GCTTTTTCTTCTTAGTCTT-3′ and 5′-TAGACTCTTAGTTACATTCTC-3′. PCR was carried out in the presence of [3H]dCTP for 30 cycles at 96 °C for 1 min/66 °C for 2.5 min. Aliquots of the PCR products were denatured at 95 °C for 5 min in formamide and applied to a 6% glycerol polyacrylamide gel (lanes 3 and 4) adjacent to aliquots of the non-denatured samples (lanes 1 and 2). The gel was subjected to electrophoresis at 300 V for 16 h at room temperature, dried, and exposed to Reflection™ NEF film for 12 h at room temperature.

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thesized only a trace amount of cholesteryl \(^{14}\text{C}\)oleate and there was no stimulation by sterols. The Tr.10–9 cells had a 2-fold increased rate of cholesteryl \(^{14}\text{C}\)oleate synthesis in the absence of sterols, and the rate rose to the same level as CHO-7 cells in the presence of sterols. Similar results were obtained in several independent experiments. None of these manipulations affected the incorporation of \(^{14}\text{C}\)oleate into \(^{14}\text{C}\)triglycerides (Table II).

To assess the sterol-regulated cleavage of SREBPs, we incubated cells in the presence of lipoprotein-deficient serum plus varying concentrations of 25-hydroxycholesterol for 16 h. Nuclear extracts were prepared and subjected to electrophoresis and immunoblotting with antibodies against SREBP-1 or -2 (Fig. 6). CHO-7 cells incubated in the absence of lipoproteins had detectable amounts of SREBP-1 and -2 in the nucleus (lane 1, upper and lower panels). The size of the protein was consistent with the known size of the transcriptionally active NH\(_2\)-terminal fragment. Addition of 0.1 \(\mu\)g/ml 25-hydroxycholesterol markedly reduced the amount of this nuclear fragment, and the protein virtually disappeared at 0.3 \(\mu\)g/ml (lanes 2 and 3). The SRD-4 cells also exhibited nuclear forms of SREBP-1 and -2, but neither protein was reduced when the cells were incubated with 25-hydroxycholesterol at concentrations as high as 1 \(\mu\)g/ml, which was 10-fold higher than the concentration that produced a detectable decrease in the CHO-7 cells (lanes 5–8). This defect in sterol-mediated regulation persisted in the Tr.10–9 cells (lanes 9–12).

The experiment of Fig. 6 indicates that the loss of sterol-mediated regulation of SREBP processing in SRD-4 cells is not caused by the loss of ACAT activity. However, the experiment does not rule out the possibility that the regulatory defect is caused by some dominant property of the mutant ACAT enzyme. To rule out this possibility, we introduced expressible cDNAs encoding wild-type or S265L mutant ACAT into 293 cells by transient transfection together with expression plasmids encoding epoite-tagged versions of SREBP-1 or -2. The cells were incubated in the absence or presence of sterols (Fig. 7). Nuclear extracts were subjected to SDS-PAGE and immunoblotting with antibodies against the epitope tag on SREBP-1 or -2. The nuclear forms of both SREBPs were down-regulated by sterols whether the cells were transfected with an empty vector (lanes 1 and 2), a vector encoding wild-type ACAT (lanes 3 and 4), or the S265L mutant of ACAT (lanes 5 and 6). Thus, there was no evidence that the S265L mutant interfered with the sterol-mediated regulation of processing of SREBPs.
DISCUSSION

The current data indicate that the SRD-4 cells contain at least three independent defects as follows: 1) a mutant allele at the ACAT locus that contains a C → T substitution, changing codon 265 from serine to leucine and producing an inactive enzyme; 2) a silent allele at the other ACAT locus that does not produce mRNA; and 3) a mutation at an unknown locus that prevents 25-hydroxycholesterol and cholesterol from inhibiting the cleavage of SREBP-1 and -2. All of these abnormalities appear to have arisen independently, and all of them act together to allow the SRD-4 cells to survive selection in the presence of 25-hydroxycholesterol.

The amino acid substitution in the ACAT protein reduces activity by two mechanisms. 1) It reduces the amount of ACAT protein, most likely because the mutant protein is unstable. 2) It severely reduces the catalytic activity of the enzyme. The first abnormality became manifest when we transfected 293 cells with a cDNA encoding the S265L mutant ACAT and found that it produced much lower levels of protein than the cDNA encoding the wild-type enzyme (Fig. 4). We were unable to detect normally in 293 cells that have been transfected with a cDNA encoding the S265L ACAT mutant.

It is straightforward to rationalize how an ACAT deficiency might help cells resist killing by 25-hydroxycholesterol (9). The sterol kills cells by suppressing the cleavage of SREBPs, thereby preventing the expression of genes encoding LDL receptors and enzymes in the cholesterol biosynthetic pathway. The resultant cholesterol deficiency is made worse because 25-hydroxycholesterol also activates ACAT, which esterifies some of the residual free cholesterol, preventing it from being used for membrane synthesis. A deficiency in ACAT would ameliorate this problem partially by abolishing esterification of cholesterol, thereby making more cholesterol available for membrane function.

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