We describe a line of mutant Chinese hamster ovary cells, designated SRD-13A, that cannot cleave sterol regulatory element-binding proteins (SREBPs) at site 1, due to mutations in the gene encoding SREBP cleavage-activating protein (SCAP) at site 1. In the SRD-13A cells, the only detectable SCAP allele encodes a truncated nonfunctional protein. In the absence of SCAP, the site 1 protease fails to cleave SREBPs, and their transcriptionally active NH2-terminal fragments cannot enter the nucleus. As a result, the cells manifest a marked reduction in the synthesis of cholesterol and its uptake from low density lipoproteins. The SRD-13A cells grow only when cholesterol is added to the culture medium. SREBP cleavage is restored and the cholesterol requirement is abolished when SRD-13A cells are transfected with expression vectors encoding SCAP. These results provide formal proof that SCAP is essential for the cleavage of SREBPs at site 1.

Sterol regulatory element-binding proteins (SREBPs) are membrane-bound transcription factors whose active fragments are released from membranes by controlled proteolysis in order to activate the synthesis of cholesterol and unsaturated fatty acids and their uptake from plasma lipoprotein. SREBP cleavage-activating protein (SCAP) is a membrane-bound regulatory protein that forms a complex with SREBPs and facilitates proteolytic release of the active fragments. SCAP contains a sterol-sensing domain that allows sterols to suppress SREBP cleavage, thereby mediating feedback suppression of lipid synthesis and uptake.

The SREBPs are tripartite proteins that are embedded in membranes of the endoplasmic reticulum (ER) and nuclear envelope in a hairpin orientation. The transcriptionally active NH2-terminal segment of ~480 amino acids projects into the cytosol. This segment contains a basic helix-loop-helix-leucine zipper sequence that allows the protein to dimerize, bind DNA, and recruit transcriptional coactivators. The NH2-terminal segment is followed by a 90-amino acid membrane attachment segment that consists of two membrane-spanning helices separated by a short hydrophilic loop of 31 amino acids that projects into the lumen of the ER and nuclear envelope. The third segment of the SREBP comprises ~590 amino acids that project into the cytosol, where they form a complex with SCAP. This segment has been called the regulatory domain.

SCAP is a polytopic membrane protein of 1276 amino acids that has two distinct domains. The NH2-terminal domain of ~730 amino acids consists of eight transmembrane helices separated by hydrophilic loops. Transmembrane helices 2–6 have been designated the sterol-sensing domain. This domain resembles sequences in three other proteins that are postulated to interact with sterols: 3-hydroxy-3-methylglutaryl CoA reductase, the Niemann-Pick C1 protein, and the developmental receptor Patched. Missense mutations at two positions within this domain of SCAP render cleavage of SREBPs insensitive to suppression by sterols. The COOH-terminal domain of SCAP (~550 amino acids) is hydrophilic and projects into the cytosol. It contains five WD repeats, which are sequences of ~40 amino acids that are found in many proteins where they mediate protein-protein interactions. The COOH-terminal WD repeat domain is the region of SCAP that forms a complex with the COOH-terminal regulatory domain of SREBPs.

Proteolytic release of the NH2-terminal fragments of SREBPs begins with the action of site 1 protease (SIP), a membrane-bound serine protease that cleaves within the luminal loop, thereby separating the two transmembrane helices. SIP is distantly related to the mammalian subtilisin-like proteases furin and the prohormone convertases. It cleaves after the leucine of the sequence Arg-Ser-Val-Leu (RSVL). Extensive, but so far indirect, experiments suggest that formation of the SCAP-SREBP complex is essential for the proteolytic cleavage of SREBPs by SIP. These experiments were performed with SREBP-2, one of the three isoforms of SREBP found in animal cells. The observations are as follows: 1) COOH-terminal truncations of SREBP-2 prevent formation of a complex with SCAP and abrogate cleavage of the SREBP; 2) overexpression of the COOH-terminal domain of either SREBP or SCAP disrupts the complex between full-length SCAP and
SREBP and abolishes cleavage at site 1; and 3) the block can be overcome in both cases by overexpression of full-length SCAP, which restores the full-length SCAP-SREBP complex (3).

Cleavage by S1P separates the two transmembrane helices of the SREBPs, but both halves remain bound to the membrane because each retains a single transmembrane helix. The NH2-terminal fragment is released from the membrane by site 2 protease (S2P), which cuts near the junction between the hydrophilic NH2-terminal fragment and the first transmembrane helix (12, 13). The cleavage site is three residues within the transmembrane helix. After cleavage by S2P, the NH2-terminal fragment leaves the membrane with three hydrophobic residues at its COOH terminus. This fragment then enters the nucleus, where it binds to sterol regulatory elements and activates transcription of more than 15 genes, whose products play roles in the biosynthesis and uptake of cholesterol and unsaturated fatty acids (1, 14).

In unraveling the SREBP proteolytic pathway, our laboratory has relied on mutant Chinese hamster ovary (CHO) cells with defects in the genes encoding S2P and S1P (13, 15). The S2P-deficient cell line was derived by mutagenesis followed by selection for cholesterol auxotrophy (16). The selection scheme is based on the method of Chang and Chang (17), who showed that cholesterol auxotrophs are resistant to the polyene antibiotic amphotericin, which kills cells by forming complexes with cholesterol in the plasma membrane. Prior to amphotericin treatment, the mutagenized CHO cells are incubated briefly in the absence of exogenous cholesterol and in the presence of a low concentration of LDL. During this period, wild-type cells activate cholesterol synthesis, and they take up LDL via LDL receptors. As a result, they maintain high levels of plasma membrane cholesterol. The auxotrophs are unable to produce cholesterol or to take it up from LDL, and their plasma membranes become relatively depleted of this sterol. Brief treatment with amphotericin kills the wild-type cells but not the auxotrophs. The mutant cells are then rescued by growth in the presence of cholesterol, the unsaturated fatty acid oleate, and a low concentration of mevalonate to supply nonsterol products (13, 15).

When this selection procedure was first employed, all of the mutants recovered from the selection had defects in the gene encoding S2P, which resides on the X chromosome. We attributed this bias to the fact that wild-type CHO cells have only one functional copy of the S2P gene. This problem was overcome when we cloned the gene for S2P by complementation (13) and used transfection techniques to produce a line of CHO cells, designated CHO/pS2P, with multiple expressed copies of the S2P cDNA (15). To isolate cholesterol auxotrophs with recessive defects at loci other than S2P, we employed a double mutagenesis protocol. The CHO/pS2P cells were first mutagenized with γ-irradiation, and potential heterozygotes were identified by incubation with fluorescently labeled LDL. The cells with low levels of LDL receptor expression were isolated with a fluorescence-activated cell sorter and subjected to a second round of γ-irradiation. Potential homozygotes were then selected for cholesterol auxotrophy using the amphotericin resistance protocol described above. This procedure yielded 12 cell lines that were auxotrophic for cholesterol. One of these, designated SRD-12B, had a defect in S1P, and these cells were used for the cloning of the S1P cDNA by complementation (11). The 11 remaining cholesterol-auxotrophic cell lines were not further characterized.

In the current studies, we characterize one of the 11 remaining amphotericin-resistant CHO cell lines. This cell line, designated SRD-13A, was found to have two defective copies of the gene encoding SCAP. As a result of the SCAP deficiency, the SRD-13A cells are unable to cleave SREBPs at site 1, and they are therefore cholesterol auxotrophs. The experiments with the SRD-13A cells provide formal genetic proof that the site 1 cleavage reaction requires SCAP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Lipoprotein-deficient serum (d = 1.215 g/ml), human LDL (d = 1.019–1.063 g/ml), sodium mevalonate, sodium oleate, and sodium compactin were prepared as described (18, 19). We obtained amphotericin B from Sigma-Aldrich; polyethylene glycol (PEG) 1500 from Roche Molecular Biochemicals (catalogue no. 783641); hydroxypropyl-β-cyclodextrin from Cyclodextrin Technologies Development, Inc. (Gainesville, FL); sterols from Steraloids, Inc.; and [3H]-cholesterol acid (51 Ci/mmol) and [2-3H]-pyruvate, sodium salt (16 mCi/mM) from NEN Life Science Products. LDL was radiolabeled with 125I as described (19). LDL labeled with 3-[(2,4-dinitrophenyl)amino]-2-octane-sulfonic acid (DNPH-SA) was prepared as described (20).

**Recombinant Plasmids**—The following expression plasmids driven by the thymidine kinase (pTK) promoter were previously described: pTK-HSV-BP-1, encoding HSV-tagged human SREBP-1; pTK-HSV-BP-2, encoding HSV-tagged human SREBP-2 (21); pTK-SCAP, encoding wild-type hamster SCAP; and pCMV-HSV-S2P, encoding HSV-tagged human S2P (13).

**Cultured Cells**—The cell lines and culture medium used are described in Table I. Cells were maintained in monolayer culture at 37 °C in a 9% CO2 incubator. CHO-7 cells are a subline of CHO-K1 cells selected for growth in lipoprotein-deficient serum (22). These cells were maintained in medium A supplemented with 5% (v/v) fetal calf lipoprotein-deficient serum. M19 cells are previously described mutant CHO cells auxotrophic for cholesterol, mevalonate, and unsaturated fatty acid (16), due to a deficiency of S2P (13). SRD-12B cells are previously described mutant CHO cells auxotrophic for cholesterol, mevalonate, and unsaturated fatty acid, due to a deficiency of S1P (11). They were isolated by an amphotericin B resistance protocol (15) and maintained in medium B. CHO/pS2P cells are CHO-7 cells expressing extra copies of a cDNA encoding S2P. Stock cultures of CHO/pS2P cells were maintained in medium A supplemented with 5% fetal calf lipoprotein-deficient serum, 2 μg/ml compactin, and 500 μg/ml G418.

**Isolation of Amphotericin B-resistant Cells Deficient in SCAP**—SRD-13A cells were isolated in the same experiment that yielded the SRD-12B cells (15). Briefly, CHO/pS2P cells were subjected to γ-irradiation mutagenesis. These cells were then grown for several days, incubated with 125I-labeled, fluorescently-labeled LDL, and subjected to γ-irradiation. Cells from a single colony from one dish were cloned by limiting dilution, and one of the resulting clones was designated SRD-13A. The SRD-13A cells were maintained in medium B at 37 °C and were subjected weekly to the amphotericin B selection protocol as described (15).

**SREBP Processing in Transfected Cells**—Cells were transfected using the FuGene 6 reagent (Roche Molecular Biochemicals). On day 0, cells were set up in 60-mm dishes at the following densities: CHO-7/pS2P, 300,000 cells; SRD-12B, 400,000 cells; and SRD-13A, 600,000 cells. On day 1, the cells were transfected with 4 μg of DNA/dish using a ratio of 12 μl of Fugene to 4 μg of DNA in medium A (without antibiotics) in a final volume of 0.2 ml. Fugene was diluted in medium A (without antibiotics) and incubated for 5 min at room temperature prior to being added dropwise to the DNA solution. This mixture was then further incubated for 15–30 min at room temperature. Plates were washed one time with 2 ml of medium A supplemented with 5% fetal calf serum and re-fed with 3 ml of the same medium. The Fugene/DNA mixture (0.2 ml) was then added to each dish. Cells were incubated at 37 °C for 16–24 h. On day 2, cells were washed one time with phosphate-buffered saline (PBS) and re-fed with either medium C or medium D. After cells received 15 μg of compactin, they were further incubated for 16 h at 37 °C, the treatment with amphotericin kills the wild-type cells but not the auxotrophs. This procedure was performed as described previously (23).
0 the cells to be tested were mixed and plated at 3.5 × 10^7 cells/well in 12-well plates and fused in the presence of polyethylene glycol. On day 2, the fused cells were trypsinized, fed, and replated onto coverslips. On day 3, the cells were fed fluorescent r-(PMCA oleate)LDL at 10 μg of protein/ml in either medium C (inducing) or medium D (suppressing). After 12 h, the cells were washed, refed medium B, fixed 4–6 h later, mounted on a slide, and then viewed by fluorescence microscopy.

**Immunoblot Analysis**—Immunoblot analysis of endogenous hamster SCAP-1 and -2 was carried out as described previously (21) with mouse monoclonal antibodies against the NH₂-terminal domains of SREBP-1 (IgG-2A4) (24) and SREBP-2 (IgG-7D4) (25), respectively. SCAP was detected with rabbit polyclonal antibody R139 (2). S1P was detected with IgG-HSV-Tag™, a monoclonal antibody directed against the glycoprotein D epitope of Herpes Simplex virus (Novagen, Inc.). The antibodies were visualized with peroxidase-conjugated, affinity-purified donkey anti-mouse or anti-rabbit IgG (Jackson Immunoresearch Laboratories) using the Enhanced Chemiluminescence Western blotting detection kit (Amersham Pharmacia Biotech). 8% SDS gels were calibrated with prestained molecular weight markers (Bio-Rad). Blots were exposed to Kodak X-Omat™ Blue XB-1 film at room temperature.

**Metabolic Assays**—The incorporation of [¹⁴C]pyruvate into cellular sterols and fatty acids (18), the incorporation of [¹⁴C]oleate into cellular cholesterol esters and triglycerides (19), and the proteolytic degradation of ¹³C-LDL (18) were measured in cell monolayers as described in Refs. 18 and 19. The protein content of cell extracts was determined by the method of Lowry et al. (27).

**DNA Sequencing of SCAP from Parental and Mutant Cells**—Total RNA was isolated from parental CHO/pS2P cells and the mutant SRD-13A cells using the RNA STAT-60 reagent (Tel-Test "B") following the manufacturer's protocol. Reverse transcriptase PCR was performed using 5 μg of total RNA as template with the SuperScript Preamplification System (Life Technologies, Inc.). First-strand cDNA synthesis was primed with the following hamster SCAP-specific oligonucleotide primer pair: 5'-TTATTACAGTCAGGAGACAACT-3' and 5'-GCACAAAA-3'. PCR products were cloned and sequenced as described above. The PCR products were cloned and sequenced as described above. The PCR products were cloned and sequenced as described above.

**RESULTS**

Table I lists the cell lines and culture media that were used in these studies. Fig. 1 shows the growth pattern of CHO/pS2P cells, which express multiple copies of the S2P cDNA, and two mutant lines designated SRD-12B and SRD-13A, which were derived from CHO/pS2P cells by two steps of mutagenesis followed by selection for low expression of LDL receptors and for cholesterol auxotrophy. Details of the two-step selection scheme are given in Ref. 15 and under “Experimental Procedures.” The SRD-12B cells were shown previously to harbor two mutant S1P genes, and their cholesterol auxotrophy can be overcome by transfection of a cDNA encoding S1P (11). The SRD-13A cells were not previously characterized. As shown in Fig. 1, the parental CHO/pS2P cells grow well in lipoprotein-deficient medium, which is low in cholesterol. The SRD-12B and SRD-13A cells grow normally only when the lipoprotein-deficient medium is supplemented with a mixture of cholesterol, mevalonate, and oleate, all of which are products of SREBP-dependent genes.

Fig. 2 shows a cell fusion assay designed to determine whether the SRD-13A cells have defects in the genes encoding S2P or S1P. In the upper panels, mixed cultures of SRD-12B and SRD-13A cells were incubated with or without the fusogen PEG, after which they were incubated with LDL that contains a fluorescent derivative of cholesterol esterified with oleate (r-(PMCA oleate)LDL). In the absence of PEG, there was no visible PMCA oleate uptake due to the absence of LDL receptors in the PEG-fused cells, LDL uptake was readily visible in the PEG-fused cells, LDL uptake was readily apparent (Fig. 2D). These data indicate that the defect in the SRD-13A cells does not reside in the gene encoding S1P. The cell fusion assay was validated by control experiments showing that homotypic fusions of SRD-13A cells with themselves did not lead to enhanced LDL uptake (data not shown). To determine whether the SRD-13A cells lies in the gene encoding S2P, we performed a similar fusion experiment with mixtures of SRD-13A cells and M-19 cells, which lack the gene for S2P. Again the fused cells showed LDL uptake (Fig. 3H), confirming that the SRD-13A cells were not defective in the S2P gene.

To study lipid synthesis in the SRD-13A cells, we incubated the cells for 24 h in the absence of cholesterol, and we then incubated them with [¹⁴C]pyruvate (Table II). The SRD-13A

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Supplemental components</th>
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<tbody>
<tr>
<td>Medium A</td>
<td>1:1 mixture of Ham’s F-12 medium and Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate.</td>
</tr>
<tr>
<td>Medium B</td>
<td>Medium A, 5% (v/v) fetal calf serum, 5 μg/ml cholesterol, 1 μg sodium mevalonate, 20 μg sodium oleate.</td>
</tr>
<tr>
<td>Medium C (inducing medium)*</td>
<td>Medium A, 5% (v/v) fetal calf lipoprotein-deficient serum, 50 μg sodium compactin, 50 μg sodium mevalonate.</td>
</tr>
<tr>
<td>Medium D (suppressing medium)*</td>
<td>Medium C, 1 μg/ml 15-hydroxycholesterol, 10 μg/ml cholesterol.</td>
</tr>
</tbody>
</table>

* Medium C and medium D contain ethanol at a final concentration of 0.2% (v/v).
cells, like the previously studied SRD-12B cells, incorporated \[^{14}\text{C}]\text{pyruvate into cholesterol at a rate that was less than 5\% of normal. On the other hand, these cells synthesized \[^{14}\text{C}]\text{fatty acids at 30–60\% of the normal rate. This result is similar to the previous measurements with M-19 cells, which lack S2P (28).}

They indicate that nuclear SREBPs are absolutely required for cholesterol synthesis, but they are only partially required for fatty acid synthesis in CHO cells.

As expected, the SRD-12B and SRD-13A cells showed marked reductions in LDL receptor activity, as determined by measurement of the high affinity degradation of \(^{125}\text{I}\text{-LDL (Table III). The cells also showed a marked reduction in the ability of LDL to stimulate incorporation of \[^{14}\text{C}]\text{oleate into cholesteryl \[^{14}\text{C}]\text{oleate (Table IV). The cells did show an increase in synthesis of cholesteryl \[^{14}\text{C}]\text{oleate when stimulated with a mixture of 25-hydroxycholesterol and cholesterol, indicating that the failure to respond to LDL was a result of the LDL receptor deficiency. The incorporation of \[^{14}\text{C}]\text{oleate into \[^{14}\text{C}]\text{triglycerides was essentially normal in the SRD-12B and SRD-13A cells (Table IV).}

To pinpoint the biochemical defect in the SRD-13A cells, we incubated the cells in the absence or presence of sterols. Nuclear and membrane extracts were subjected to SDS-PAGE and blotted with antibodies against various components of the processing system (Fig. 3). The SRD-13A cells were compared with the S1P-deficient SRD-12B cells and the parental CHO/pS2P cells. As shown in Fig. 3A, nuclear extracts from the CHO/pS2P cells contained the mature form of SREBP-1 and SREBP-2 when the cells were incubated in the absence (lane 1) but not the presence (lane 2) of sterols. Nuclear extracts from the SRD-12B and SRD-13A cells did not show detectable amounts of SREBP-1 or SREBP-2 under either condition (lanes 3–6). The membrane fraction of the CHO/pS2P and SRD-12B cells contained abundant amounts of the precursor forms of SREBP-1 and SREBP-2. The SRD-13A cells had reduced amounts of both precursors.

Membranes from the CHO/pS2P cells contained abundant...
amounts of SCAP (Fig. 3B) that were not affected by the absence or presence of sterols (lanes 1 and 2). The SRD-12B cells had reduced amounts of SCAP when incubated in the absence of sterols (lane 3), and the amount rose when sterols were present (lane 4). The SRD-13A cells lacked detectable SCAP when incubated either in the absence or presence of sterols (lanes 5 and 6).

The CHO/pS2P and SRD-13A cells had normal amounts of S1P as visualized by blotting with anti-S1P (Fig. 3B, middle blot). Moreover, both cell lines had a similar distribution of the three processed forms of S1P (A, B, and C) (26). As expected, the SRD-12B cells lacked all immunodetectable S1P (Fig. 3B, middle panel).

Table II

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<thead>
<tr>
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<tbody>
<tr>
<td>CHO/pS2P</td>
<td>2.6</td>
<td>8.9</td>
<td>5.6</td>
</tr>
<tr>
<td>SRD-12B</td>
<td>0.13</td>
<td>3.0</td>
<td>5.6</td>
</tr>
<tr>
<td>SRD-13A</td>
<td>0.09</td>
<td>3.0</td>
<td>3.0</td>
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</table>

Table III

<table>
<thead>
<tr>
<th>Cell line</th>
<th>125I-LDL degraded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>CHO/pS2P</td>
<td>1017</td>
</tr>
<tr>
<td>SRD-12B</td>
<td>135</td>
</tr>
<tr>
<td>SRD-13A</td>
<td>116</td>
</tr>
</tbody>
</table>

On day 0, monolayers of cells were set up for experiments in medium B at 6–10 × 10⁴ cells/60-mm dish. On day 1, the cells were washed with PBS and switched to medium A supplemented with 5% fetal calf lipoprotein-deficient serum. On day 2, the cells were washed twice with PBS and refed medium A containing 5% fetal calf lipoprotein-deficient serum and 2 μg [14C]pyruvate (2.7 dpm/μmol). After incubation at 37°C for 2.5 h, the cells were harvested for measurement of the cellular content of [14C]cholesterol and [14C]fatty acids. A blank value, representing the amount of [14C]pyruvate incorporated into [14C]cholesterol (0.03–0.04 nmol/h/mg) and [14C]fatty acids (0.01–0.06 nmol/h/mg) in each cell line that was incubated for 2 h at 4°C was subtracted from each value. Each value is the mean of triplicate incubations.

To determine whether the SCAP deficiency is responsible for the failure of SRD-13A cells to cleave SREBPs, we transfected SRD-13A cells with expression vectors encoding HSV-tagged SREBP-1a (Fig. 4A) or SREBP-2 (Fig. 4B) with increasing amounts of a plasmid encoding SCAP (Fig. 4). The cells were incubated in the absence or presence of sterols, and the amounts of membrane-bound and nuclear SREBPs were measured. As expected, in the absence of SCAP there was no nuclear SREBP (lane 1). As the amount of SCAP plasmid increased, there was a corresponding increase in the amounts of nuclear SREBP-1a and SREBP-2 in the cells incubated in the absence of sterols (lanes 3, 5, and 7). When sterols were present, the amounts of the nuclear SREBPs were reduced (lanes 4, 6, and 8). Thus, expression of SCAP rescues sterol-regulated cleavage of SREBPs in the SRD-13A cells.

If the SCAP cDNA restores site 1 cleavage in SRD-13A cells, then it should also restore growth in the absence of cholesterol. The experiment of Fig. 5 indicates that this was the case. The experiment of Fig. 6 was designed to determine whether permanent restoration of SCAP expression would restore sterol-regulated processing of endogenous SREBP-2 in SRD-13A
Cells were set up as described in the legend to Table II. On day 1, the cells were washed with PBS and switched to medium A supplemented with 5% fetal calf lipoprotein-deficient serum, 50 μM compactin, and 50 μM sodium mevalonate. On day 2, the cells were switched to 2 ml of Dulbecco's modified Eagle's medium (without glutamine) containing 2 mg/ml bovine serum albumin and 10 μM compactin and either no additions, sterol (1 μg/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol), or LDL (100 μg of protein/ml). After incubation for 5 h at 37 °C, cells were pulsed for 2 h with 0.2 μM [14C]oleate/albumin (10 dpm/nmol), after which the cells were harvested and fractionated as described under “Experimental Procedures.” Aliquots of membrane and nuclear extract fractions (20 μg protein/lane) were subjected to SDS-PAGE and immunoblot analysis with 5 μg/ml R139, a rabbit polyclonal antibody directed against SCAP. The filters were exposed to film at room temperature for 1–2 s.

**Fig. 4.** Restoration of sterol regulation of cleavage of HIV-tagged SREBP-1 (A) and SREBP-2 (B) in SRD-13A cells transiently transfected with pTK-SCAP. On day 0, mutant SRD-13A cells were set up at 5 × 10^5 cells/60-mm dish in medium B. On day 1, cells were transfected with 2 μg/dish of either pTK-HSV-BP-1 (A) or pTK-HSV-BP-2 (B) together with the indicated amount of pTK-SCAP. On day 2, the cells were switched to inducing medium C (Sterols) or suppressing medium D (+ Sterols) and incubated for 16 h as described in Fig. 3. Aliquots of membrane and nuclear extract fractions (20 μg of protein/lane) were subjected to SDS-PAGE and immunoblot analysis with either 0.2 μg/ml of IgG-HSV-Tag or 5 μg/ml R139, a rabbit polyclonal antibody directed against SCAP. The filters were exposed to film at room temperature for 1–2 s.

**Fig. 5.** Restoration of growth of SRD-13A cells by transfection with pTK-SCAP. On day 0, cells were plated at 6 × 10^5 cells/60-mm dish in medium B. On day 1, the cells were transfected with either 4 μg/dish of empty vector or 0.25 μg/dish of pTK-SCAP together with 3.75 μg/dish of empty vector as described under “Experimental Procedures.” On day 2, the cells were washed once with PBS and refed with either medium B containing cholesterol, mevalonate, and oleate or medium A supplemented with 5% fetal calf lipoprotein-deficient serum. Cells were refed every 2–3 days. On day 14, the cells were washed, fixed in 95% ethanol, and stained with crystal violet.

For this purpose, we obtained three independent clones of SRD-13A cells that were permanently transfected with pTK-SCAP and had regained the ability to grow in the absence of cholesterol (see Fig. 6). The three cloned cell lines are designated A, B, and C. To induce SREBP processing, we incubated the cells overnight in suppressing medium that contained a mixture of 25-hydroxycholesterol plus cholesterol. The cells were then washed and switched to fresh medium with or without cycloheximide, an agent that efficiently removes sterols from cells (29). The medium also contained ALLN, which inhibits the degradation of the nuclear form of SREBP-1 (30). As controls, we studied the parental CHO/pS2P cells and the SCAP-deficient SRD-13A cells. In the presence of cycloheximide, nuclear

### Table IV

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cholesterol [14C]oleate formed</th>
<th>[14C]Triglycerides formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO/pS2P</td>
<td>None 1800 (3.7)p</td>
<td>None 7624 (4.2)p</td>
</tr>
<tr>
<td>SRD-12B</td>
<td>With sterols 6689 (3.7)p</td>
<td>With sterols 7624 (4.2)p</td>
</tr>
<tr>
<td>SRD-13A</td>
<td>With LDL 115</td>
<td>With LDL 145 (1.3)</td>
</tr>
</tbody>
</table>

* Denotes fold stimulation by the addition of sterols or LDL.
extracts from the CHO/pS2P cells contained the nuclear form of SREBP-2 as determined by immunoblotting (Fig. 6, lane 1). The nuclear protein was not detected when cyclodextrin was omitted (lane 2). As expected, the SRD-13A cells lacked nuclear SREBP-2 under either condition (lanes 3 and 4). All three of the permanently transfected SCAP-expressing SRD-13A cells showed normal amounts of nuclear SREBP-2, and this was abolished when cyclodextrin was omitted (lanes 5–10). The upper panel of Fig. 6 also shows that restoration of SCAP expression restored normal levels of the precursor form of SREBP-2 in the membrane fraction of SRD-13A cells. The bottom panel of Fig. 6 confirms that SCAP expression was restored in lines A–C as determined by blotting with anti-SCAP.

We next conducted a series of experiments designed to assess the nature of the genetic defect that leads to SCAP deficiency in the SRD-13A cells. Fig. 7 shows a Northern blot, comparing the amounts of SCAP mRNA in the CHO/pS2P cells, the SRD-12B cells, and the SRD-13A cells. Quantification by exposing the filters to a Fujix BAS1000 Bio-Imaging Analyzer revealed that the amount of SCAP mRNA was reduced by 55% in the SRD-13A cells when compared with the other two cell lines. The mRNA that remained in the SRD-13A cells was approximately the same size as the SCAP mRNA in the CHO/pS2P cells (~4.2 kilobases).

To determine whether the residual SCAP mRNA in SRD-13A cells could encode a functional SCAP protein, we amplified the mRNA by reverse transcriptase-PCR, inserted the amplified fragments into a cloning vector, and sequenced nine independent clones. All of the clones showed a 14-bp deletion that disrupted the reading frame at codon 133, leading to an altered sequence of amino acids that terminates at position 160 (Fig. 8A). If this truncated protein were produced, it would contain only one of the eight membrane-spanning helices, and it would lack both the sterol-sensing domain and the WD repeat region (Fig. 8A). Such a protein could not be functional.

To determine the cause of the 14-bp deletion in the SCAP mRNA, we subjected the relevant region of genomic DNA to PCR, and we sequenced 16 clones derived from wild-type cells and SRD-13A cells. All of the SRD-13A sequences had a single point mutation that changed a C to a G. This creates a new splice site that is 14 bp upstream from the normal splice site (Fig. 8B). The resulting mRNA has a deletion of 14 bp. Apparently, the new splice site is used exclusively, because all of the cellular mRNA shows the 14-bp deletion.

The fact that all of the genomic clones had the same point mutation in the SRD-13A cells...

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**Fig. 7.** Northern blot analysis of total RNA from parental CHO cells and mutants auxotrophic for cholesterol. Total RNA was prepared from the indicated cell lines using RNAStat60 (TelTest B) according to the manufacturer's instructions. Aliquots of total RNA (20 µg/lane) were subjected to electrophoresis in a denaturing (formaldehyde) gel (32) and transferred to a Hybond N+ membrane (Amersham Pharmacia Biotech) by capillary blotting. Replicate blots were probed with one of the following 32P-labeled probes: 2.8-kilobase fragment from the 5'-end of hamster SCAP (4) or 1.2-kilobase cDNA encoding rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control (33). Hybridization was carried out for 60 min at 65 °C in RapidHyb buffer (Amersham Pharmacia Biotech) at 1 × 106 cpm/ml. The filters for SCAP and GAPDH were exposed at -70 °C for 14 h and 20 min, respectively.

**Fig. 8.** Identification of mutation in SRD-13A cells. A, sequence of SCAP transcripts. The upper diagram shows a model of the membrane topology of SCAP (5). Below the model are the nucleotide and deduced amino acid sequences of the wild-type SCAP transcript and the mutant SCAP transcript from SRD-13A cells. The mutant sequence was determined by reverse transcriptase-PCR analysis and sequencing of nine independent clones as described under “Experimental Procedures.” B, sequence of genomic DNA. The sequence of the genomic DNA from the region encoding the deleted portion of the transcript from mutant SRD-13A cells in A was determined from PCR-generated clones. Sixteen clones from the wild-type and mutant cells were sequenced. The mutation resulting from the C to G change in codon 133 (denoted by an asterisk) creates a new consensus splice donor site in the SRD-13A DNA (34), utilization of which yields a transcript with the observed 14-bp deletion shown in A.
mutation suggests that the SRD-13A cells are either homozygous for the point mutation shown in Fig. 8, or else they have a deletion in the second copy of the gene that removes at least this region. Genomic Southern blots failed to show abnormal restriction fragments in the SRD-13A cells (data not shown). Thus, if there is a deletion in the second copy of the SCAP gene, the deletion may be large enough to encompass the whole gene plus substantial amounts of flanking DNA.

**DISCUSSION**

The current data provide compelling genetic evidence that SCAP is absolutely required in order for the site 1 protease to cleave SREBPs. The mutant SRD-13A cells, which fail to produce functional SCAP mRNA, are unable to cleave endogenous SREBPs (Fig. 3) or SREBPs that have been overexpressed as a result of transfection (Fig. 4). Cleavage is restored when wild-type SCAP is expressed as a result of transient or stable transfection (Fig. 4 and 6, respectively). As a result of the failure to cleave SREBPs, the SRD-13A cells fail to synthesize normal amounts of cholesterol, and they require cholesterol, oleate, and mevalonate for growth. The auxotrophies are abolished when SCAP expression is restored (Fig. 5).

An unexpected finding was the low levels of SREBP precursors in the SRD-13A cells (Figs. 3 and 6). This phenomenon appears to be secondary to the SCAP deficiency, since the levels of SREBP precursors were restored to normal in the three lines of SRD-13A cells that were permanently transfected with pTK-SCAP (Fig. 6).

Co-immunoprecipitation experiments indicate the vast majority of SREBPs in CHO cells are present in a complex with SCAP (2). If the COOH terminus of SREBPs cannot form a complex with SCAP, the proteins may be rapidly degraded. Such degradation must occur through a pathway that does not release the NH₂-terminal fragments of SREBPs into the cytosol.

Interestingly, we did not observe a deficiency of SREBP-2 when the precursor was produced in SRD-13A cells as a result of transient transfection with the pTK-SREBP-2 cDNA (Fig. 4). It is possible that overexpression of SREBP-2 saturates the degradative process, allowing the precursor to accumulate. Resolution of this issue will require further studies of the synthesis and turnover of SREBPs in the SRD-13A cells.

The mechanism by which SCAP facilitates S1P activity is not yet known. Previous data provide evidence that the SCAP-SREBP complex remains sequestered in the ER when cells are overloaded with sterols. When sterols are depleted, the carbohydrate chains of SCAP become resistant to endoglycosidase H, suggesting that the SCAP-SREBP complex has moved to the Golgi (31). It is possible that SREBP must leave the ER in order to be cleaved by S1P and that the function of SCAP is to control this movement according to the sterol content of the cell. The availability of the SCAP-deficient SRD-13A cells should permit testing of this hypothesis by transfection of wild-type and mutant SCAP in an attempt to correlate site 1 cleavage with the ability of mutant forms of SCAP to leave the ER.

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**REFERENCES**