Isolation of Cholesterol-requiring Mutant Chinese Hamster Ovary Cells with Defects in Cleavage of Sterol Regulatory Element-binding Proteins at Site 1*

Robert B. Rawson, Dong Cheng, Michael S. Brown, and Joseph L. Goldstein

From the Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, Texas 75235

The synthesis and uptake of cholesterol requires transcription factors designated sterol regulatory element-binding proteins (SREBPs). SREBP are bound to membranes in a hairpin orientation with their transcriptionally active NH2-terminal segments facing the cytosol. The NH2-terminal segments are released from membranes by two-step proteolysis initiated by site 1 protease (S1P), which cleaves in the luminal loop between two membrane-spanning segments. Next, site 2 protease (S2P) releases the NH2-terminal fragment of SREBP. The S2P gene was recently isolated by complementation cloning using Chinese hamster ovary cells that require cholesterol for growth, due to a mutation in the S2P gene. A similar approach cannot be used for S1P because all previous cholesterol auxotrophs manifest defects in S2P, which is encoded by a single copy gene. To circumvent this problem, in the current studies we transfected Chinese hamster ovary cells with the S1P cDNA, assuring multiple copies. We mutagenized the cells, selected for cholesterol auxotrophy, and identified two mutant cell lines (SRD-12A and -12B) that fail to cleave SREBPs at site 1. Complementation analysis demonstrated that the defects in both cell lines are recessive and noncomplementing, indicating a mutation in the same gene. These cells should now be useful for expression cloning of the sterol-regulated S1P gene.

Studies in tissue culture cells have defined a feedback regulatory system that controls the synthesis and uptake of cholesterol and fatty acids. The central agents are membrane-bound transcription factors designated as sterol regulatory element-binding proteins (SREBPs) (reviewed in Ref. 1). These proteins, which average 1,150 amino acids in length, consist of three segments. The NH2-terminal segment of ~480 amino acids is a transcription factor of the basic-helix-loop-helix-leucine zipper family. This is followed by a membrane attachment segment consisting of two membrane-spanning sequences separated by a 31-amino acid hydrophilic loop that projects into the lumen of the endoplasmic reticulum and nuclear envelope. The COOH-terminal segment of ~580 amino acids performs a regulatory role. The SREBPs are inserted into the membranes of the endoplasmic reticulum and nuclear envelope in a hairpin fashion with the NH2-terminal and COOH-terminal segments projecting into the cytosol (2).

In sterol-depleted cells, a two-step proteolytic process releases the NH2-terminal segments (designated nSREBP) from the membranes, allowing them to enter the nucleus, where they stimulate transcription of multiple genes encoding enzymes of cholesterol synthesis, including HMG-CoA synthase, HMG-CoA reductase, farnesyl diphosphate synthase, squalene synthase, and others (1, 3–6). The nSREBPs also enhance transcription of genes encoding enzymes of fatty acid and triglyceride biosynthesis (acyl-CoA carboxylase, fatty acid synthase, stearoyl-CoA desaturase-1 and -2, glycerol-3-phosphate acyltransferase) (5–10) and for the low density lipoprotein (LDL) receptor (1, 6). When sterols build up in cells, the proteolytic release of nSREBPs is blocked, and transcription of target genes declines (1). This system assures cells an adequate supply of cholesterol and unsaturated fatty acids while avoiding overaccumulation.

A molecular dissection of the two-step cleavage process is currently under way. The first cleavage occurs at site 1, between the Leu and Ser of the sequence RSVLS, which is located in the middle of the hydrophilic luminal loop (11). Site 1 cleavage occurs in sterol-depleted cells, and it is abolished by sterol excess. This cleavage requires SREBP cleavage-activating protein (SCAP), a polytopic membrane protein with a long cytosolically exposed COOH-terminal segment that contains 5 “WD” repeats, which generally mediate protein-protein interactions (12, 13). The COOH-terminal segment of SCAP forms a tight complex with the COOH-terminal domain of SREBPs, an interaction that is required for site 1 cleavage (13). In addition to activating cleavage, current evidence indicates that SCAP serves as the sterol sensor in this system. Five of the eight membrane-spanning segments of SCAP resemble sequences in the membrane-attachment domain of HMG-CoA reductase (12). In HMG-CoA reductase, this domain mediates rapid degradation when cellular sterols rise (12, 14), indicating that it serves as a sterol sensor. In SCAP, the membrane attachment domain also mediates sterol regulation. Indeed, missense mutations within this domain abolish the ability of sterols to suppress the cleavage of SREBPs at site 1 (12, 15). Sequences resembling the sterol-sensing domain are found in two other proteins that are postulated to interact with sterols, the Niemann-Pick C1 protein, which is required for intracellular transport of cholesterol (16), and Patched, which is the receptor for the signaling morphogen, Hedgehog, the only protein known to have a covalently attached cholesterol (17).

Cleavage at site 1 separates SREBPs into two halves, each of which remains membrane-bound, due to the retention of a membrane-spanning segment (1). Release of the nSREBP requires a second protease that cleaves at site 2, which is a
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Leu-Cys bond that is located within the NH₂-terminal membrane-spanning segment (18). This cleavage requires the sequence DRSR, which defines the border between the cytosolic sequence and the membrane-spanning sequence (2, 18). The cleaved Leu-Cys bond is located three residues to the COOH-terminal side of the DRSR (18). Following cleavage at this site, the nSREBP leaves the membrane with three hydrophobic residues attached. The site 2 protease is not regulated directly by sterola, but it can act only after site 1 cleavage has separated the SREBPs into two halves.

Our laboratory recently employed complementation cloning techniques to isolate a cDNA encoding the site 2 protease (19). The key was the availability of a mutant line of Chinese hamster ovary (CHO) cells designated M19, which require cholesterol and unsaturated fatty acids for growth (20). These cells cannot produce their own cholesterol because they are unable to release nSREBPs from membranes (21). Biochemical studies showed that these cells cleave SREBPs at site 1, but they are unable to cleave at site 2. Under conditions of sterol deprivation, the M19 cells accumulate a membrane-bound intermediate form of nSREBP that is the product of site 1 cleavage (21). The defect in the M19 cells was corrected by transfection of two cell lines, designated SRD-12A and -12B, that have lost two functional copies of the S1P gene. We succeeded in isolating two cell lines, designated SRD-12A and -12B, that have lost the activity of S1P. In this paper, we describe the isolation and characterization of these cells, which should prove useful for expression cloning of the S1P cDNA.

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 (24, 25). We obtained amphotericin B from Sigma; polyethylene glycol (PEG) 1500 from Boehringer Mannheim (catalogue no. 783641); sterols from Steraloids, Inc.; and [1,2-¹⁴C]oleic acid (112 mCi/mmol) from NET Life Science Products. LDL was radiolabeled with [¹²⁵I]I as described (25). LDL labeled with 3-rectanglemethyl-23,24-dinor5-cholen-22-olate-3β-yl oleate (PMCA oleate, a fluorescent analogue of cholesteryl ester) was prepared as described (26).

Recombinant Plasmids—The following plasmids were previously described: pCMV-SREBP-1A460, a plasmid encoding a amino acids 1–460 of human SREBP-1a (27); pTK-HSV-BP2, encoding HSV-tagged human SREBP-2 (28); pTK-HSV-BP2 (R519A), encoding HSV-tagged human SREBP-2 containing a mutation that abolishes cleavage at site 1 (28); pTK-HSV-BP2(DRSR -> AS), encoding HSV-tagged human SREBP-2 containing a mutation that abolishes cleavage at site 2 (28); pCMV-SCAP(D443N), encoding a dominant-positive mutant version of hamster SCAP (12); and pCMV-HSV-S2P, encoding HSV-tagged human S2P (19).

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 (29). These cells were maintained in medium A supplemented with 5% (v/v) fetal calf lipoprotein-deficient serum. M19 cells are previously described mutant CHO-K1 cells auxotrophic for cholesterol, mevalonate, and unsaturated fatty acid isolated by an amphotericin B resistance protocol (20). These cells were maintained in medium B. ld-7 cells are mutant CHO-K1 cells that lack functional LDL receptors (30). CHO/opoS2P cells were isolated by transfection of CHO-7 cells with plasmid pCMV-HSV-S2P (which contains the neo gene) followed by selection for growth in medium A supplemented with 5% fetal calf lipoprotein-deficient serum and 750 µg/ml G418. G418-resistant colonies were cloned by limiting dilution and screened for expression of the human S2P cDNA by immunoblot analysis of urea-solubilized membrane fractions (19) (described under “Immunoblot Analysis”). Stock cultures of CHO/opoS2P cells were maintained in medium A supplemented with 5% fetal calf lipoprotein-deficient serum, 2 µg compin, and 500 µg/ml G418.

Mutagenesis and Isolation of Amphotericin B-resistant Cells Deficient in Site 1 Cleavage—Two strategies were used to isolate two mutant cell lines deficient in site 1 cleavage, designated SRD-12A and -12B cells. SRD-12A cells were isolated as follows. On day 0, CHO/opoS2P cells were harvested from stock cultures by trypsinization, centrifuged, washed, and resuspended in phosphate-buffered saline (PBS). The cell suspension was divided into six aliquots of 1 × 10⁶ cells/ml per aliquot, which were each exposed to 750 rads of γ-irradiation from a ¹³⁷Cs source using a Mark 1 irradiator (J. L. Shepherd Co., San Fernando, CA). The cells were immediately plated at 5 × 10⁶ cells/100-mm dish in medium B. Cells were grown at 34 °C for 6 days, with fresh medium B added every 2 days. On day 5, the cells were shifted to 39 °C for 5 h prior to being washed once with PBS and refed with medium A supplemented with

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10% newborn calf serum and 10 μg of protein/ml of human LDL. On day 6, the cells were washed once with PBS and refed with medium A supplemented with 1% newborn calf lipoprotein-deficient serum and 50 μg/ml amphotericin B in dimethyl sulfoxide (final concentration of dimethyl sulfoxide was 0.05% (v/v)). The cells were incubated 3–5 h at 39 °C, washed twice with PBS, refed medium B, and returned to 34 °C. The above cycle of feeding, temperature shift, and amphotericin B selection was repeated on days 14, 22, 28, and 36. One colony survived these multiple rounds of selection. It was recovered with a cloning cylinder, replated, and designated SRD-12A cells. Subsequent studies showed that the mutant phenotype in the SRD-12A cells was not temperature-sensitive, and the cells were therefore grown routinely at 37 °C.

SRD-12B cells were isolated as follows: On day 0, CHO/pS2P cells were harvested from stock cultures as described above. Two aliquots of 1 × 10⁶ cells each were suspended in 1 ml of PBS and mutagenized with 800 rads of γ-irradiation as described above. Cells were plated at 5 × 10⁵ cells/100-mm dish in medium B. The medium was changed every 2 days. On day 6, the cells were washed and refed medium containing 5% fetal calf lipoprotein-deficient serum, 50 μM sodium mevalonate, 50 μM sodium compactin, and 10 μg of protein/ml of PMCA oleate-labeled LDL. On day 7, the cells were harvested by trypsinization, washed, and resuspended in PBS. The cells were then sorted on a FACStarPLUS fluorescence-activated cell sorter (Becton Dickinson Immucytometry Systems, San Jose, CA) using an excitation wavelength of 360 nm and an emission wavelength of 380 nm. Parameters were set to recover cells whose fluorescence intensity was in the bottom 10% of all the cells, a region overlapping the fluorescence of cells not incubated with PMCA oleate-labeled LDL. Of ~2.5 × 10⁷ cells sorted, ~1.25 × 10⁶ were recovered in this manner. The sorted cells were plated at 2.5 × 10⁵ cells/100-mm dish in medium B containing 10% fetal calf serum. Cells were refed medium B containing 10% fetal calf serum every 2 days. On day 14, the cells were split 1:3. On day 21, the cells were washed and harvested as above, and five aliquots of ~1 × 10⁷ cells/ aliquot were mutagenized with 750 rads of γ-irradiation and plated in 100-mm dishes at ~5 × 10⁶ cells/dish. Cells were subjected to multiple cycles of amphotericin B selection as described above for the SRD-12A cells. Of the 100 original dishes, 12 contained colonies, ranging from 1 to 5 per dish. Each colony was replated and subjected to additional rounds of amphotericin B selection, and the most vigorous colony was cloned by limiting dilution and designated SRD-12B cells.

The SRD-12A and -12B cells were maintained in medium B at 37 °C and were subjected weekly to a cycle of amphotericin B selection as described above.

**Luciferase Reporter Assay**—On day 0, each cell line was set up at 1 × 10⁵ cells/22-mm well in medium A supplemented with 5% fetal calf lipoprotein-deficient serum and 20 μM sodium oleate. On day 1, each well was transfected by the calcium phosphate method with the MBS kit in a final volume of 0.2 ml as described above. The following plasmids were transfected: 0.8 μg of plasmid encoding luciferase under control of a promoter/enhancer consisting of three tandem copies of repeat 2 + 3 from the LDL receptor promoter plus the adenosvirus E1b TATA box (pSRE-luciferase) (12); 0.05 μg of pCMVβ, a plasmid encoding β-galactosidase (Stratagene); and 0.05 μg of either pCMV empty vector or pCMV-SREBP-1a460, a plasmid encoding amino acids 1–460 of human SREBP-1a (27). After incubation for 3 h at 35 °C, the cells were washed with PBS and switched to medium C (inducing medium) or medium D (suppressing medium) (see Table 1). After further incubation at 37 °C for 20 h, the cells were lysed with 0.2 ml of 1× reporter lysis buffer (Promega), and aliquots from each well were assayed for luciferase activity (40 μl) and β-galactosidase activity (15 μl) as described (19). The amount of luciferase activity in transfected cells was normalized to the amount of β-galactosidase activity to correct for transfection efficiency.
Immunoblot Analysis—Immunoblot analysis of endogenous hamster SREBP-1 and –2 was carried out as described previously (28) with mouse monoclonal antibodies against the NH2-terminal domains of SREBP-1 (IgG-2A4) (31) and SREBP-2 (IgG-7D4) (32), respectively. SCAP was detected with monoclonal antibody IgG-9D5 (33). Epitope-tagged proteins were 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 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]acetate into cellular sterols and fatty acids (24), the incorporation of [14C]oleate-albumin into cellular cholesteryl esters and triglycerides (25), and the proteolytic degradation of 125I-LDL (25) were measured in cell monolayers as described. The protein content of cell extracts was determined by the method of Lowry et al. (34).

RESULTS

As the wild-type parental strain for these studies, we chose CHO-7 cells, which are a clone of CHO-K1 cells that have a high rate of cholesterol synthesis as a result of adaptation to growth in lipoprotein-deficient serum (29). In the first step, we transfected these cells with a cDNA encoding S2P under control of the cytomegalovirus promoter in a vector encoding resistance to the antibiotic G418. We selected a clone of G418-resistant cells that produces high levels of S2P as determined by immunoblotting (data not shown). We interpreted this finding to indicate that these cells had integrated multiple functional copies of the S2P cDNA and that they therefore would require several mutations in order to lose S2P activity. This clone was designated as CHO/pS2P, and it was used as the parental line for the mutagenesis studies.

The CHO/pS2P cells were mutagenized with γ-irradiation, and two different protocols were used to isolate mutants with a defect in S1P. In the first approach, designed to enrich for temperature-sensitive mutants, the cells were grown at 34 °C and shifted to 39 °C for 3–5 h in medium containing newborn calf serum supplemented with additional LDL (10 μg of protein/ml). Under these conditions, the cells will become cholesterol-deficient only if they have a simultaneous block in cho-

**FIG. 1.** Growth of parental and mutant CHO cells in the presence and absence of nutrients. On day 0, cells were plated at 5 × 10⁶ cells/100-mm dish in medium B. On day 1, the cells were washed once with PBS and refed every 2–3 days with one of the following media: medium B containing 5% fetal calf serum, cholesterol, mevalonate, and oleate (left column; see Table I); medium A containing 5% fetal calf lipoprotein-deficient serum (right column). On day 14, the cells were washed, fixed in 95% ethanol, and stained with crystal violet.

| Cell Line       | Additions to Medium | Cholesterol Mevalonate Olate | None |
|-----------------|---------------------|-----------------------------|------|
| CHO/ pS2P       |                     |                             |      |
| M19 (S2P deficient) |                   |                             |      |
| SRD-12A (S1P deficient) |               |                             |      |
| SRD-12B (S1P deficient) |               |                             |      |
TABLE II
Incorporation of $^{14}$Cacetate into $^{14}$Ccholesterol and $^{14}$C-fatty acids in parental CHO/pS2P cells and mutant cells auxotrophic for cholesterol

| Cell line | $^{14}$C-Cholesterol (pmol/h per mg protein) | $^{14}$C-Fatty acids (pmol/h per mg protein) |
|-----------|---------------------------------|---------------------------------|
| CHO/pS2P  | 749                             | 5,411                           |
| SRD-12A   | 3.5                             | 1,251                           |
| SRD-12B   | 4.5                             | 1,769                           |

TABLE III
Receptor-mediated degradation of $^{125}$I-LDL in parental CHO/pS2P cells and mutant cells auxotrophic for cholesterol

| Cell line | High affinity degradation of $^{125}$I-LDL (ng/5 h per mg protein) |
|-----------|--------------------------------------------------|
| CHO/pS2P  | 477                                              |
| SRD-12A   | 5                                                |
| SRD-12B   | 0                                                |

Mutant CHO Cells Defective in Site 1 Cleavage of SREBPs

On day 0, monolayers of cells were set up for experiments in medium B at $6 \times 10^4$ cells (CHO/pS2P cells) or $7.5 \times 10^4$ cells (SRD-12A and SRD-12B cells) per 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 0.5 mM $[^{14}]$Cacetate (25 dpm/pmol). After incubation at 37 °C for 2 h, the cells were harvested for measurement of the cellular content of $[^{14}]$Ccholesterol and $[^{14}]$C-fatty acids. A blank value representing the amount of $[^{14}]$Cacetate incorporated into $[^{14}]$Ccholesterol (13–45 pmol/h per mg) and $[^{14}]$C-fatty acids (7–25 pmol/h per 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.

On day 0, the cells were set up at $1.5 \times 10^5$ cells/100-mm dish in medium B as described under “Experimental Procedures.” On day 2, the cells were washed twice with prewarmed medium A and then switched to inducing medium C (+ sterols) or suppressing medium D (+ sterols). After incubation for 16 h at 37 °C, the dishes received N-acetyl-leucinyl-leucinyl-norleucinal to achieve a final concentration of 25 μg/ml. After incubation for 2.5 h, the cells were harvested and fractionated as described previously (21). Aliquots of the membrane and nuclear extract fractions (150 μg protein/lane) were subjected to SDS-polyacrylamide gel electrophoresis. Immunoblot analysis was carried out with 5 μg/ml monoclonal IgG-2A4 for SREBP-1 (A) or 5 μg/ml monoclonal IgG-7D4 for SREBP-2 (B). The filters were exposed to film for 2 and 5 min in A and B, respectively. P, I, and N denote the precursor, intermediate, and nuclear cleaved forms of SREBP, respectively.

FIG. 2. Sterol-regulated processing of SREBP-1 and SREBP-2 in parental CHO cells and mutants auxotropic for cholesterol.

Choledlsterol biosynthesis and in LDL receptor activity. The cells were then treated with amphoterin B, and the survivors were returned to 34 °C in medium B, which is supplemented with mevalonate, and oleate (see Table I). The temperature shift/amphoterin selection protocol was repeated four times on subsequent days. Of $6 \times 10^4$ cells subjected to this selection, only one clone survived. This clone was designated SRD-12A (sterol regulatory defective-12A). Subsequent studies showed that the phenotype was not temperature-sensitive, and the cells were therefore grown permanently at 37 °C in medium B.

Because of the low efficiency of the above procedure, we devised a stepwise selection method that was designed to enrich initially for cells lacking one copy of the S1P gene and subsequently for cells lacking both copies. In this protocol, the mutagenized CHO/pS2P cells were incubated with LDL that was reconstituted with a fluorescent derivative of cholesterol esterified with oleate (PMCA oleate) (26). When cells were incubated with this lipoprotein at low concentrations, their lysosomes become fluorescent, but only when they express functional LDL receptors. After this incubation, the cells were subjected to fluorescence-activated cell sorting, and we selected the population whose fluorescence was in the lower 10% of all sorted cells. This population should include those rare cells that had a partial deficiency of LDL receptors, due to a 50% deficiency of S1P activity. The sorted cells were grown in large numbers in medium B and then subjected to a second round of mutagenesis and several rounds of amphoterin B selection in order to select cells that had a block in cholesterol synthesis in addition to LDL receptor deficiency. This procedure yielded more amphoterin-resistant colonies than did the temperature shift protocol. The first mutagenesis was performed with $2 \times 10^7$ cells, and we ended up with more than 10 amphoterin-resistant clones. The most vigorous clone was maintained in medium B and is designated SRD-12B.

Fig. 1 shows a series of stained Petri dishes in an experiment designed to compare the growth requirements of the SRD-12A and -12B cells, the parental CHO/pS2P cells, and M19 cells, which lack S2P. The parental CHO/pS2P cells grew in medium containing lipoprotein-deficient serum as well as in medium B, which is supplemented with mevalonate, and oleate. In contrast, all three mutant cell lines grew only in the presence of the supplements.

To compare the abilities of the CHO/pS2P cells and SRD-12 cells to synthesize cholesterol, we first incubated both cell lines in medium B for 24 h in order to equalize their exposure to cholesterol, oleate, and mevalonate. It was then necessary to deplete the cells of cholesterol in order to activate the processing of SREBPs. For this purpose, the cells were switched into medium containing only lipoprotein-deficient serum. After 24 h, all of the cells appeared healthy. We then pulsed the cells with $[^{14}]$Cacetate and measured its incorporation into $[^{14}]$Ccholesterol and $[^{14}]$C-fatty acids (Table II). The rate of cholesterol
synthesis in the SRD-12A and -12B cells was less than 1% of the rate in the parental CHO/pS2P cells. The synthesis of fatty acids averaged about 30% of the rate in the CHO/pS2P cells. These results resemble those reported previously in M19 cells, which lack S2P (35).

To measure LDL receptor activity, we incubated the SRD-12 cells and the CHO/pS2P cells in medium B for 24 h and then switched them to inducing medium, which contains lipoprotein-deficient serum, the HMG-CoA reductase inhibitor compactin, and a low level of mevalonate (250 μM), which supplies substrate for nonsterol products (36). After 24 h, we added 125I-LDL and measured the rate of high affinity degradation, which is dependent on uptake by LDL receptors (Table III). The SRD-12A and -12B cells degraded 125I-LDL at less than 1% of the rate seen in CHO/pS2P cells.

The combined failure to synthesize cholesterol and to take up LDL strongly suggests that the SRD-12A and -12B cells have a deficiency of nSREBPs. To test this hypothesis directly, we incubated the cells in inducing medium with or without sterols and then performed SDS-polyacrylamide gel electrophoresis on membrane fractions and nuclear extracts (Fig. 2). The proteins were transferred to nitrocellulose and blotted with antibodies against the NH2-terminal segments of SREBP-1 and SREBP-2 (Fig. 2A) or SREBP-2 (Fig. 2B). When the parental CHO/pS2P cells were incubated in the absence of sterols, the precursor forms of SREBP-1 and -2 were found in the membrane fraction, and the NH2-terminal segments were found in the nuclear extracts (lane 1). When sterols were added, the nSREBPs disappeared, and there was a corresponding increase in the precursors (lane 2). In the sterol-deprived M19 cells, there was no detectable nSREBP in the nuclear extracts (lane 3). Instead, there was an intermediate form of SREBP-1 and SREBP-2 in the membrane fraction that reflects the product of site 1 cleavage. This intermediate form disappeared in the sterol-treated cells, reflecting the inhibition of site 1 cleavage (lane 4). In the sterol-deprived SRD-12A and -12B cells, we found normal amounts of the SREBP precursors in the membrane fractions, but there was no detectable cleavage product either in the membrane fraction or in the nuclear extract fraction (lanes 5 and 7). Despite the failure to detect SREBP cleavage products in the SRD-12A and -12B cells, we did detect an increase in the amounts of the precursor forms when sterols were added (lanes 6 and 8). The potential significance of this finding is discussed below.

To further demonstrate that the failure of the SRD-12A and -12B cells to process SREBPs is not due to a defect either in the SREBPs themselves or in SCAP, we performed experiments in cells that were transfected with cDNAs encoding epitope-tagged versions of wild-type and mutant SREBP-2 (Fig. 3). The cells were incubated in the presence of fetal calf serum to suppress cleavage and transfected with a cDNA encoding the D443N mutant form of SCAP, which overcomes sterol suppression of the S1P. Processing of transfected SREBP-2 was followed by SDS-polyacrylamide gel electrophoresis and immunoblotting with an antibody against the HSV epitope. In CHO-7 cells transfected with a cDNA encoding wild-type SREBP-2, we detected the nuclear form only when SCAP was cotransfected (Fig. 3, A and B, lane 3). The nuclear form was not detected when the transfected SREBP-2 contained the R519A mutation, which abolishes site 1 cleavage (lane 5). The DRSR mutant of

**Fig. 3.** Proteolytic processing of HSV-tagged SREBP-2 in transiently transfected SRD-12A (A) and SRD-12B (B) cells in the absence or presence of SCAP(D443N). Cells were set up on day 0 and transiently transfected on day 1 with the indicated plasmid encoding wild-type (WT) or mutant HSV-tagged SREBP-2 (3 μg/dish) together with either pCMV-SCAP(D443N) (0.5 μg/dish) or empty vector (0.5 μg/dish) as described under “Experimental Procedures.” Cells in lane 1 were transfected with 3.5 μg of DNA consisting of the appropriate empty vectors. On day 2, the cells were harvested and fractionated as described under “Experimental Procedures.” Aliquots of membrane and nuclear extract fractions (30 μg of protein/lane) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis with 0.5 μg/ml of IgG-HSV-Tag¢ or 5 μg/ml IgG-9D5, a mouse monoclonal antibody directed against SCAP. Filters were exposed for 5 and 20 s for anti-HSV-Tag and anti-SCAP immunoblots, respectively, P, I, and N denote the precursor, intermediate, and cleaved nuclear forms of SREBP, respectively.

**Fig. 4.** Transcription of SRE-luciferase reporter gene is stimulated by transfected dominant positive SREBP-1a in mutant cells. Cells were set up for experiments, cotransfected as described under “Experimental Procedures” with the SRE-luciferase reporter plasmid, the β-galactosidase plasmid pCMVβ, and either pCMV empty vector (A) or pCMV-SREBP-1a460 (B) as indicated. After transfection, the cells were switched to either inducing (No sterols) or suppressing (+ sterols) medium, incubated for 20 h, harvested, and assayed for luciferase activity as described under “Experimental Procedures.” Results are plotted as the mean of four independent transfections. Individual values for each transfected cell line varied by <15% of the mean value.
SREBP-2 also failed to generate a nuclear form, but it did generate a membrane-bound intermediate form that was SCAP-dependent (lane 7), reflecting the fact that this mutant form can be cleaved at site 1 but not site 2. The SRD-12A cells (panel A) and the SRD-12B cells (panel B) failed to produce the nuclear form when transfected with the cDNA encoding wild-type SREBP-2 (lane 9), nor did they generate the intermediate form when the cells produced the DRSR3AS mutant (lane 13). This failure was observed although the cells overproduced exogenous SCAP (lower panels), confirming that their defect was not due to a defect in SCAP. We also showed that the SRD-12A and -12B cells failed to process transfected epitope-tagged SREBP-2 even when grown in full inducing medium in the absence of sterols (data not shown). This result, together with the data of Figs. 2 and 3, rules out the possibility that the defect in the SRD-12A and -12B cells lies in the genes for the SREBPs.

The failure to generate nSREBPs in the SRD-12A and -12B cells is expected to produce a defect in transcription from promoters that contain a sterol regulatory element and therefore depend on nSREBPs for high level transcription. To test this prediction, we transfected the SRD-12A and -12B cells with cDNAs encoding a reporter gene (luciferase) under control of a promoter containing multiple copies of tandem repeats 1 and 2 from the LDL receptor promoter. Repeat 1 contains a weak...
binding site for the general transcription factor Sp1, but it drives transcription at a very low rate unless there is simultaneous binding site for the general transcription factor Sp1, but it was no effect of sterols (Figs. 5, 6).

If the transcriptional defect in the SRD-12A and -12B cells is attributable to the failure to process SREBPs, then the defect should be relieved if we cotransfect a cDNA encoding a truncated nSREBP that lacks the membrane attachment domain and enters the nucleus without a requirement for proteolysis. Indeed, as shown in Fig. 4B, the production of luciferase in the SRD-12A and -12B cells was equal to that in the CHO/pS2P cells when all three cell lines were transfected with a cDNA encoding nSREBP-1a. As expected, none of the cells showed inhibition by sterols.

The immunoblotting data suggest that the SRD-12A and -12B cells have a defect in site 1 cleavage, whereas the M19 cells have a defect in site 2 cleavage. If both disorders are recessive, we would predict that the two cell lines should complement each other; i.e. in cell fusion experiments the M19 cells should supply the site 1 cleavage enzyme, and the SRD-12A or -12B cells should supply the site 2 cleavage activity. As a result, the SREBP target genes should be transcribed in a normal sterol-regulated fashion. To test this hypothesis, we fused SRD-12B cells with M19 cells and monitored LDL receptor activity by fluorescence microscopy after incubation of the cells with PMCA oleate LDL. When the two cell lines were simply cocultured and incubated without the polyethylene glycol fusogen, there was no visible uptake of the fluorescent LDL, and there was no effect of sterols (Figs. 5, B and D). When the cocultured cells were fused by incubation with PEG, many of the multinucleated heterokaryons took up the fluorescent LDL, indicating that they were expressing LDL receptors (Fig. 5F). Uptake was suppressed by incubation with sterols (Fig. 5H), confirming that it was attributable to the restoration of regulated processing of SREBPs.

The fluorescence microscopy complementation assay was applied pairwise to all of the cell lines used in the current studies and also to ldl-A7 cells, which have normal SREBP processing but fail to take up PMCA oleate LDL because of a defect in the gene encoding the LDL receptor. The results are summarized in Table IV. In control experiments, homotypic fusions of CHO cells to CHO cells and CHO/pS2P cells to CHO/pS2P cells preserved LDL uptake, whereas homotypic fusions of the mutant cell lines failed to restore LDL uptake. As expected, uptake of PMCA oleate LDL was restored when the ldl-A7 cells were fused to any of the SREBP processing mutants (M19, SRD-12A, or SRD-12B cells) or when M19 cells were fused to either the SRD-12A or -12B cells. Importantly, uptake was not restored when SRD-12A cells were fused to SRD-12B cells, indicating that both of these cells have a mutation in the same gene.

**Discussion**

The experiments described in this paper represent a significant advance in the 25-year quest for a molecular understanding of the pathway by which cells regulate the cholesterol content of their membranes. A crucial element of this pathway is the sterol-regulated protease that cleaves SREBPs at site 1 (1, 11). This protease has eluded molecular characterization, because attempts to establish in vitro assays of its activity have failed. This failure is perhaps understandable, because the target of the protease resides in the luminal loop of an endoplasmic reticulum protein. Moreover, the protease requires the participation of a complex polytopic membrane protein, SCAP, which interacts with sequences of the substrate that are on the opposite (cytosolic) side of the membrane (13). No protease with similar requirements has yet been assayed biochemically in any system. The alternate approach is to use the techniques of somatic cell genetics to clone the gene for the site 1 protease by complementation, as was done for the site 2 protease (19). Implementation of this strategy has not been possible up to now because all of the mutants in SREBP processing isolated to date have involved the gene for S2P, apparently because it is a single copy gene in CHO cells.

The production of mutants in S1P activity was achieved by the following sequential steps: 1) the isolation of cholesterol auxotrophic CHO cells with mutations in the gene encoding S2P (20, 38); 2) the cloning of the human S2P gene through genetic complementation of the CHO cell mutants (19); 3) the use of the S2P cDNA to produce a CHO cell line (CHO/pS2P) with multiple copies of the coding sequences for S2P; and 4) the use of amphotericin selection and a fluorescence-activated cell sorter to isolate extremely rare cholesterol auxotrophic mutants of the CHO/pS2P cells whose defect could be traced to a deficiency of S1P activity.

At present, we have studied two cell lines, SRD-12A and -12B, that have defects in the site 1 cleavage reaction. The SRD-12A cells were produced by a single round of mutagenesis followed by several rounds of selection with amphotericin. Only one clone survived this procedure out of $6 \times 10^7$ that were mutagenized. The SRD-12B cells were produced by two rounds of mutagenesis with an intervening cell sorting step to select for cells that have a partial reduction in LDL receptor activity. The two-step procedure produced at least 10 clones of mutant cells out of $2 \times 10^7$ that were mutagenized. We do not know whether these were all derived independently or whether they represent duplicate isolations of the SRD-12B cells.

Genetic complementation tests revealed that the mutant phenotypes in both the SRD-12A and -12B cells are recessive, suggesting that they result from loss-of-function mutations.

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**Table IV**

| Cell fusions                  | Without PEG | With PEG |
|------------------------------|-------------|----------|
|                              | Without sterols | With sterols | Without sterols | With sterols |
| CHO × CHO                    | +            | +         | +                | +            |
| CHO/pS2P × CHO/pS2P          | +            | +         | +                | +            |
| M19 × M19                    | -            | -         | -                | -            |
| ldl-A7 × ldl-A7              | -            | -         | -                | -            |
| SRD-12A × SRD-12A            | -            | -         | -                | -            |
| SRD-12B × SRD-12B            | -            | -         | -                | -            |
| M19 × ldl-A7                 | -            | -         | +                | +            |
| SRD-12A × ldl-A7             | -            | -         | +                | +            |
| SRD-12B × ldl-A7             | -            | -         | +                | +            |
| SRD-12A × M19                | -            | -         | +                | +            |
| SRD-12B × M19                | -            | -         | +                | +            |
| SRD-12A × SRD-12B            | -            | -         | +                | +            |

*+, uptake of fluorescent LDL as shown in Fig. 5, E and F; -, no detectable uptake of fluorescent LDL.*
Moreover, the two clones fail to complement each other, suggesting that the mutations involve the same gene. It is highly likely that this gene encodes S1P, although we cannot rule out the possibility that it encodes a protein that is absolutely required for the action of S1P. Importantly, the SRD-12A and -12B cells grow normally when supplied with cholesterol, mevalonate, and oleate, suggesting that their defect is restricted to the SREBP processing pathway. We have not yet studied the individual requirements for each of these supplements, but it seems likely that the S1P-deficient cells like the S2P-deficient cells, will require both cholesterol and unsaturated fatty acids.

One possible candidate for the defect in the SRD-12A and -12B cells is the gene encoding SCAP. Previous studies, performed with cDNAs encoding dominant-negative truncated SCAP, showed that the SCAP/SREBP interaction is absolutely required for the site 1 cleavage reaction (13). However, defective site 1 cleavage was not corrected when the SRD-12A or -12B cells were transfected with a cDNA encoding a superactive form of SCAP, i.e. the D443N mutant (Fig. 3), thereby ruling out a SCAP defect in the SRD-12A and -12B cells.

Although the SRD-12A and -12B cells did not show evidence for cleavage of SREBP-1 or SREBP-2 in the absence of sterols, they nevertheless showed an increase in the amount of uncleaved precursor when sterols were added (Fig. 3). At present, we do not have a definitive explanation for this observation. In cells that lack S1P, the SREBP precursor may be rapidly degraded when sterols are absent. The addition of sterols may stabilize the precursor, perhaps by stabilizing its partner, SCAP. In studies to be published elsewhere, we have observed that sterols increase the amount of immunodetectable SCAP in cultured cells.5

The processing of SREBPs in this study was assessed by immunoblot analysis, which reflects the protein level at steady state. The deficiency of nSREBPs in SRD-12A and -12B cells could theoretically be explained by an abnormally rapid acceleration in the turnover of nSREBPs within the nucleus as well as by decreased site 1 cleavage. This possibility was excluded by the experiment in which the mutant cells were transfected with the cDNA encoding truncated SREBP-1a (Fig. 4). Transfected nSREBP-1a bypassed the block in S1P and stimulated transcription of a sterol regulatory element-driven reporter gene to the same extent in SRD-12A, SRD-12B, and wild-type cells, thus ruling out the possibility of accelerated degradation of nSREBPs in the mutant cells.

The availability of the recessively mutated SRD-12A and -12B cells should now permit the complementation cloning of a normal copy of the defective S1P gene through the use of transfection approaches similar to those used for the cloning of S2P.

Acknowledgments—We thank Lisa Beatty and Rosa Shamloo for invaluable help with cell culture; Gloria Brunschede and Debra Morgan for excellent technical assistance; and Angie Mobley for superb help with fluorescence-activated cell sorting analysis.

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