Overexpression of Membrane Domain of SCAP Prevents Sterols from Inhibiting SCAP-SREBP Exit from Endoplasmic Reticulum*

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SCAP (SREBP cleavage-activating protein) forms a complex with sterol regulatory element-binding proteins (SREBPs) and escorts them from the endoplasmic reticulum (ER) to the Golgi complex where proteases release transcriptionally active segments of SREBPs, which enter the nucleus to activate lipid synthesis. The NH2-terminal segment of SCAP contains eight transmembrane helices, five of which (TM2–6) comprise the sterol-sensing domain. This domain responds to sterols by causing the SCAP-SREBP complex to be retained in the ER, preventing proteolytic release and reducing transcription of lipogenic genes. Here, we use transfection techniques to overexpress a segment of SCAP containing transmembrane helices 1–6 in hamster and human cells. This segment does not interfere with SCAP-SREBP movement to the Golgi in the absence of sterols, but it prevents sterols from suppressing this movement. This block is abolished when SCAP(TM1–6) contains a point mutation (Y298C) that is known to abolish the activity of the sterol-sensing domain. We interpret these findings to indicate that sterols cause the SCAP-SREBP complex to bind to an ER retention protein through an interaction that involves the sterol-sensing domain. The SCAP(TM1–6) segment competes with the SCAP-SREBP complex for binding to this putative retention protein, thereby liberating the SCAP-SREBP complex so that it can move to the Golgi despite the presence of sterols. These studies provide a potential mechanistic explanation for the ability of sterols to block SCAP-SREBP movement from the ER and thereby to control lipid synthesis in animal cells.

The gated movement of sterol regulatory element-binding proteins (SREBPs) from endoplasmic reticulum (ER) to the Golgi complex controls the rate of lipid synthesis in animal cells (1). SREBPs are membrane-bound transcription factors whose NH2-terminal domains must be released proteolytically to enter the nucleus. For proteolysis to take place, the SREBPs must be transported from their site of synthesis in the ER to their site of proteolysis in the Golgi complex (2). This transport is accomplished by SCAP (SREBP cleavage-activating protein), a membrane-bound protein that forms a complex with SREBPs in the ER and escorts them to the Golgi complex.

SREBPs are tripartite membrane proteins of ~1150 amino acids in length (1). The NH2-terminal domain of ~480 amino acids contains a basic-helix-loop-helix-leucine-zipper motif that allows it to bind DNA and activate transcription. This domain is followed by a membrane attachment domain of ~80 amino acids consisting of two transmembrane helices separated by a short hydrophilic loop that projects into the lumen of the ER. The COOH-terminal domain of ~590 amino acids performs a regulatory function. The SREBPs are oriented in the membrane in a hairpin fashion with the NH2- and COOH-terminal domains projecting into the cytoplasm (3).

SCAP is a polytopic membrane protein with two distinct domains (4). The NH2-terminal domain of ~550 amino acids consists of alternating hydrophobic and hydrophilic sequences that are believed to form eight membrane-spanning helices. The COOH-terminal domain of ~725 amino acids is hydrophilic and projects into the cytoplasm. This domain contains five copies of a WD-40 repeat sequence that is found in many proteins and mediates protein/protein interactions (5). Indeed, immediately after its synthesis, SCAP forms a complex with SREBPs that is mediated by an interaction of the WD-40 repeat domain of SCAP and the COOH-terminal regulatory domain of the SREBPs (6, 7).

When cells are depleted of sterols, the SCAP-SREBP complex travels from the ER to the Golgi, where it encounters two proteases that act in sequence to release the NH2-terminal domain of SREBP into the cytoplasm (2, 8). The first enzyme, designated Site-1 protease, cleaves the SREBP in the luminal loop, thereby separating the two transmembrane helices (9). This allows Site-2 protease to cleave the first transmembrane helix within the plane of the membrane, releasing the NH2-terminal domain with three hydrophobic amino acids attached (10). This domain travels to the nucleus where it activates transcription of multiple genes involved in the synthesis of cholesterol and fatty acids as well as their uptake from plasma lipoproteins through the low density lipoprotein receptor (1).

Movement of SREBP to the Golgi absolutely requires SCAP. In SCAP-deficient mutant cell lines, the SREBPs fail to move to the Golgi complex, and they are not released proteolytically from membranes. As a result, SCAP-deficient cells are cholesterol auxotrophs (11). The SCAP-SREBP transport process is controlled by cholesterol. When sterols build up in cells, the SCAP-SREBP complex fails to bud from ER membranes and it never reaches the Golgi. As a result, the synthesis of cholesterol and fatty acids is reduced. This sterol-regulated transport mechanism modulates the cholesterol content of cell membranes.

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1 The abbreviations used are: SREBP, sterol regulatory element-binding protein; SCAP, SREBP cleavage-activating protein; ALLN, N-acetyl-leucinal-leucinal-norleucinal; CMV, cytomegalovirus; endo H, endoglycosidase H; ER, endoplasmic reticulum; HEK-293, human embryonic kidney 293 cells; HSV, herpes simplex virus; PCR, polymerase chain reaction; TM1–6, transmembrane helices 1–6; Xp, Xpress epitope tag; kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis.
The sterol-sensing function in the SREBP system has been traced to a portion of the membrane domain of SCAP encompassing transmembrane helices 1–6 (TM1–6). This region contains sequences that are shared with three other proteins that are postulated to interact with sterols (12). This segment has therefore been termed “the sterol-sensing domain” (13). Point mutations at two conserved residues within this domain (Y298C and D443N) abolish the sterol-sensing function of SCAP (12, 14). These mutant forms of SCAP form complexes with SREBPs that are transported to the Golgi normally, but they can no longer be blocked by sterols. As a result, cells bearing these SCAP mutations overproduce cholesterol and fail to shut-off cholesterol synthesis when intracellular sterol levels rise.

A crucial question relates to the mechanism by which SCAP is retained in the ER in sterol-overloaded cells. Two mechanisms must be considered. The first model postulates that the SCAP-SREBP complex leaves the ER constitutively and that sterols cause the complex to bind to a molecule, presumably a protein, that actively retains it in the ER. The alternative hypothesis states that ER retention of the complex represents the constitutive state. For the SCAP-SREBP complex to leave the ER, it must interact with a protein that carries it to the Golgi. This interaction would occur only in sterol-depleted cells.

The current studies were designed to distinguish between these two possibilities. For this purpose, we transfected cells with cDNAs encoding a portion of the transmembrane domain of SCAP that includes the sterol sensor. Overexpression of this domain abolished the ability of sterols to suppress SCAP movement and SREBP cleavage. This effect was not observed when the transmembrane domain containing the Y298C mutation. These findings support the model in which the SCAP-SREBP complex is retained in the ER through an interaction between the transmembrane domain and an unidentified retention protein. The overexpressed transmembrane domain competes for this interaction and allows the SCAP-SREBP complex to move constitutively even in the presence of sterols.

**EXPERIMENTAL PROCEDURES**

Materials—We obtained monoclonal HSV-Tag antibody (IgG3) from Novagen; monoclonal anti-Xpress antibody (IgG3) from Invitrogen; rat anti-mouse horseradish peroxidase-conjugated antibody (ε-chain) from ICN; 2-hydroxypropyl-β-cyclodextrin from Cycloexdrin Technologies Development, Inc.; and trypsin (catalog number LS003744) from Worthington Biochemical Co. All other reagents were obtained from Worthington Biochemical Co. All other reagents were obtained from Worthington Biochemical Co.

**Construction of Plasmids**—Expression vectors pTK, pTK-HSV-BP2, pTK-HSV-BP2(R519A), pTK3-SCAP, and pCMV-SCAP have been previously described (6, 12, 16). All expression vectors described below contain sequences that are shared with three other proteins that are postulated to interact with sterols (12). This segment has therefore been termed “the sterol-sensing domain” (13). Point mutations at two conserved residues within this domain (Y298C and D443N) abolish the sterol-sensing function of SCAP (12, 14). These mutant forms of SCAP form complexes with SREBPs that are transported to the Golgi normally, but they can no longer be blocked by sterols. As a result, cells bearing these SCAP mutations overproduce cholesterol and fail to shut-off cholesterol synthesis when intracellular sterol levels rise.

**RESULTS**

**Fig. 1** shows a diagram of the membrane topology of SCAP, denoting the two segments of SCAP that were produced by the
cDNAs used in these studies. The positions of the various epitopes, the three N-linked glycosylation sites, and the Y298C mutation are shown. The sterol-sensing domain of SCAP comprises transmembrane helices 2–6 (shaded in Fig. 1).

Fig. 2 shows an experiment in which cultured human cells were transfected with a cDNA encoding SREBP-2 with an epitope tag at the NH2 terminus. The cells were also transfected with cDNAs encoding a portion of the membrane domain of SCAP (transmembrane helices 1–6) tagged with an Xpress (XP) epitope. The encoded protein contained either the wild-type sequence or the Y298C mutation. The cells were incubated in the absence of sterols or in the presence of increasing concentrations of a mixture of 25-hydroxycholesterol and cholesterol, after which nuclear extracts and membrane fractions were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with antibodies against either the HSV-tag on SREBP-2 or the Xpress-tag on the truncated SCAP. When the cells expressing HSV-SREBP-2 alone were incubated in the absence of sterols, the SREBP was cleaved, and the NH2-terminal segment was found in the nuclear extract (top panel, lane 2). The addition of sterols abolished cleavage and the nuclear segment disappeared (lanes 3–5). Expression of the truncated wild-type XP-SCAP(TM1–6) did not affect SREBP cleavage in the absence of sterols (lane 6), but it prevented suppression of cleavage by sterols (lanes 7–9). When the XP-SCAP(TM1–6) protein bore the Y298C mutation, it lost the ability to block sterol suppression (lanes 10–13). The bottom panel of Fig. 2 shows that the wild-type and Y298C-truncated SCAP proteins were expressed at equal levels in the cells.

The experiment of Fig. 3A shows that the abolition of sterol suppression required the first six transmembrane helices of SCAP. Transfection of cDNAs encoding smaller segments that included transmembrane segments 1–5 failed to abolish sterol suppression (lanes 10 and 11). Suppression was abolished only when the construct contained transmembrane domains 1–6 (lanes 12 and 13).

In the experiment of Fig. 3B, we transfected cells with a cDNA encoding a truncated form of SCAP that extends from amino acids 1 to 1195. This construct encodes all eight transmembrane helices plus the bulk of the cytosolic WD-40 domain (see Fig. 1). However, it is truncated at a position that is 80 amino acids from the COOH terminus of the protein. Recent studies have shown that this truncated protein does not interact with SREBPs as determined by a failure of coimmunoprecipitation, nor does it restore SREBP cleavage to SCAP-deficient cells. As seen before, when we transfected the cells with HSV-SREBP-2 alone, sterols suppressed cleavage (Fig. 3B, lanes 2–4). When we cotransfected SCAP(1–1195), sterol suppression was abolished (lanes 5–7). The effect of this truncated SCAP was eliminated when we deleted transmembrane helices 3–6 (lanes 8–10), supporting the idea that these transmembrane helices were required for this effect. The bottom panel of Fig. 3B shows that comparable amounts of the SCAP(1–1195) construct and the transmembrane 3–6 deletion were expressed in the cells.

The membrane domain of SCAP contains three N-linked carbohydrate chains that are attached to loops that project into the ER lumen (see Fig. 1) (4). In sterol-depleted cells, SCAP moves to the Golgi complex where the carbohydrates are processed by enzymes that render them resistant to digestion by endo H (8). When cells are grown in the presence of sterols, the SCAP-SREBP complex is trapped in the ER, and the N-linked sugars remain in their unprocessed, endo H-sensitive state. To detect the changes in electrophoretic mobility upon endo H treatment, we first treated intact membrane vesicles with trypsin to reduce the size of the glycosylated fragment thereby permitting clear discrimination between glycosylated and nonglyco-

3 A. Nohturfft, J. L. Goldstein, and M. S. Brown, unpublished observations.
Fig. 3. Prevention of sterol-mediated inhibition of SREBP-2 cleavage: requirement for transmembrane segments 1–6 of SCAP. On day 0, HEK-293 cells were set up for experiments as described under “Experimental Procedures.” On day 2, the cells were transfected with the indicated plasmids: A, 2.5 μg of pTK-HSV-SREBP-2 (lanes 2–13); 2 μg of pCMV-SCAP(1–307, TM 1–6) (lanes 4 and 5); pCMV-SCAP(1–383, TM 1–4) (lanes 6 and 7); pCMV-SCAP(1–346, TM 1–5) (lanes 8 and 9); pCMV-SCAP(1–415, TM 1–5) (lanes 10 and 11); and pCMV-SCAP(1–448, TM 1–6) (lanes 12 and 13). The total amount of DNA was adjusted to 4.5 μg/dish by addition of pTK mock vector and/or pcDNA3.1HisC mock vector. After transfection, the cells were incubated in medium B with 0.2% ethanol in the absence or presence of 1 μg/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol (sterols). After incubation for 16 h, the cells were harvested and membrane fractions were prepared as described under “Experimental Procedures.” All membranes were incubated with trypsin, proteolysis was stopped by addition of trypsin inhibitor, and the SDS-solubilized membranes were then treated with endo H. The samples were subjected to SDS-PAGE and immunoblot analysis with 2 μg/ml anti-SCAP polyclonal antibody (IgG-R139). Filters were exposed to film for 15 s.

Numbers 0–2 on the right denote differentially glycosylated forms of the trypsin-resistant SCAP fragment containing the corresponding numbers of N-linked oligosaccharides.

Fig. 4. Expression of SCAP(TM 1–6) partially blocks the ability of sterols to trap endogenous SCAP in ER as judged by endo H sensitivity. On day 0, HEK-293 cells were set up for experiments as described under “Experimental Procedures.” On day 3, the cells were transfected with the indicated plasmids as follows: 2.5 μg of pTK-HSV-SREBP-2 (lanes 2–7); 2 μg of pCMV-Xp-SCAP(1–448) (lanes 4 and 5); and 2 μg of pCMV-Xp-SCAP(1–448, Y298C) (lanes 6 and 7). The total amount of DNA was adjusted to 4.5 μg/dish by addition of pTK mock vector and/or pcDNA3.1HisC mock vector. After transfection, the cells were incubated in medium B with 0.2% ethanol in the absence or presence of 1 μg/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol (sterols). After incubation for 16 h, the cells were harvested and membrane fractions were prepared as described under “Experimental Procedures.” All membranes were incubated with trypsin, proteolysis was stopped by addition of trypsin inhibitor, and the SDS-solubilized membranes were then treated with endo H. The samples were subjected to SDS-PAGE and immunoblot analysis with 2 μg/ml anti-SCAP polyclonal antibody (IgG-R139). Filters were exposed to film for 15 s. Numbers 0–2 on the right denote differentially glycosylated forms of the trypsin-resistant SCAP fragment containing the corresponding numbers of N-linked oligosaccharides.
absence or presence of sterols (compare lanes 6 and 7 with lanes 2 and 3). This experiment demonstrates that the wild-type SCP(TM1–6) segment prevents sterols from blocking the movement of endogenous SCAP from ER to Golgi.

The results of Figs. 2–4 support the hypothesis that the SCP(TM1–6) segment interacts with a putative protein that retains endogenous SCAP in the ER in the presence of sterols. This interaction displaces the endogenous SCAP-SREBP complex from the retaining protein, thereby allowing it to travel to the Golgi even in the presence of sterols.

The experiment of Fig. 5 was conducted to determine whether functional SCAP activity could be reconstituted in SCAP-deficient cells by coexpression of SCP(TM1–6) (residues 1–448) plus a second cDNA encoding the remainder of the protein (residues 449–1276). When the cells were transfected with a cDNA encoding wild-type SREBP-2 in the absence of SCAP, there was no processing to the nuclear form (upper panel, lane 2). Transfection of the SCP(449–1276) segment...
did not restore cleavage (lane 3), nor did transfection of a cDNA encoding the NH₂-terminal segment with either the wild-type sequence (lane 4) or the Y298C mutation (lane 5). Cleavage was restored only when we expressed SCAP(449–1276) together with SCAP(1–448) containing either the wild-type sequence (lane 6) or the Y298C mutation (lane 7). The specificity of this effect was documented by the demonstration that no combination of SCAP segments could achieve cleavage of the R519A mutant of SREBP-2, which is not recognized by Site-1 protease (lanes 8–13) (9, 16). The two bottom panels of Fig. 5 show that the various segments of SCAP were expressed in the transfected cells as determined by immunoblotting with the anti-Xp, which recognizes the NH₂-terminal segment, or anti-SCAP, which recognizes the COOH-terminal segment.

The positive result in Fig. 5 suggests that the NH₂- and COOH-terminal segments of SCAP are able to interact so as to restore SCAP activity even though they are expressed as separate proteins. To study this interaction directly, we performed a communoprecipitation experiment (Fig. 6). SCAP-deficient SRD-13A cells were transfected with cDNAs encoding the COOH-terminal segment of SCAP(449–1276) together with the NH₂-terminal segment (1–448) containing either the wild-type or Y298C sequence. Membrane extracts were immunoprecipitated with anti-Xp, which recognizes only the NH₂-terminal segment. The supernatant and pellet fractions from the immunoprecipitations were subjected to SDS-PAGE and blotted with anti-Xp or with anti-SCAP, which detects the COOH-terminal segment. As shown in the bottom panel of Fig. 6, a fraction of the COOH-terminal segment was found in the pellet after precipitation with the antibody against the NH₂-terminal segment (lanes 3–6), indicating that these two segments were physically associated. The communoprecipitation was the same in the absence and presence of sterols.

If the NH₂-terminal and COOH-terminal segments of SCAP can physically interact when transfected into cells, then they should be able to restore the growth of SCAP-deficient SRD-13 cells, which are auxotrophic for cholesterol (11). The experiment of Fig. 7, in which SRD-13 cells were transfected with various SCAP segments and grown for 14 days in the absence of cholesterol, indicates that this was indeed the case.

**DISCUSSION**

The current experiments demonstrate that overexpression of a portion of the transmembrane domain of SCAP (transmembrane helices 1–6) abolishes the ability of sterols to cause the retention of the SCAP-SREBP complex in the ER. Instead, the SCAP-SREBP complex moves to the Golgi complex where the carbohydrates of SCAP are processed to an endo H-resistant form and where SREBP is cleaved by the Site-1 and Site-2 proteases to liberate the transcriptionally active NH₂-terminal segment. When the SCAP(TM1–6) segment contains the Y298C mutation, its ability to block sterol repression of SCAP-SREBP movement is markedly reduced.

The simplest hypothesis to explain these results postulates that the SCAP(TM1–6) segment competes with the endogenous SCAP-SREBP complex for binding to a protein that retains the complex in the ER in the presence of sterols. This hypothesis is supported by the observation that the Y298C mutation in the SCAP(TM1–6) segment abolishes this effect. The Y298C mutation in full-length SCAP has already been shown to render the protein unresponsive to sterols (12). Thus, SCAP(TM1–6)/Y298C does not compete for the retention protein either because tyrosine 298 of SCAP is essential to bind sterols, which in turn triggers binding to the retention protein, or, alternatively, SCAP(TM1–6)/Y298C may continue to bind sterols, but it cannot bind to the retention protein. Either of these mechanisms would explain the failure of SCAP/Y298C to be regulated by sterols as well as the finding that SCAP(TM1–6)/Y298C does not compete with endogenous SCAP-SREBP for the retention protein in the presence of sterols.

SCAP(TM1–6) includes the sterol-sensing domain (TM2–6) that is shared with three other proteins (reviewed in Ref. 13). It will be important to learn whether the sterol-sensing domains in these proteins interact with the same protein that retains SCAP and whether SCAP(TM1–6) will interfere with the actions of these proteins. One of these proteins is 3-hydroxy-3-methylglutaryl-coenzyme A reductase, an enzyme of cholesterol biosynthesis that is bound to ER membranes (14). Sterols accelerate the degradation of this enzyme in an action that requires the sterol-sensing domain (18, 19). We are currently conducting experiments to determine whether overexpression of SCAP(TM1–6) will interfere with the ability of sterols to accelerate the degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase.

Another important finding in these studies is that the NH₂-terminal and COOH-terminal domains of SCAP, expressed separately, can reconstitute the SREBP transport function of SCAP in SCAP-deficient hamster cells. This reconstitution was apparently attributable to the ability of these two segments to form a complex that was susceptible to coimmunoprecipitation (Fig. 6). The COOH-terminal segment that we employed contains three distinct regions: 1) TM segments 7 and 8; 2) the large intraluminal loop that connects these segments; and 3) the cytoplasmic WD-repeat domains. One or more of these regions must be capable of binding to the TM1–6 segment, thereby restoring the function of SCAP.

If the proposed mechanism of SCAP(TM1–6) activity is correct, this protein segment should provide a powerful tool for isolating the postulated ER retention protein with which it interacts.

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