

Topology of SREBP Cleavage-activating Protein, a Polytopic Membrane Protein with a Sterol-sensing Domain*

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The NH₂-terminal fragments of sterol regulatory element-binding proteins (SREBPs) are released from endoplasmic reticulum membranes by proteases whose activities depend upon SREBP cleavage-activating protein (SCAP), a polytopic endoplasmic reticulum membrane protein. The activity of SCAP is inhibited by sterols, which appear to interact with the polytopic membrane domain of SCAP. Here, we use protease protection and N-linked glycosylation site-mapping techniques to define the topology of the eight membrane-spanning domains of SCAP. The data indicate that the NH₂ terminus and COOH terminus of SCAP face the cytosol. The long intraluminal loops after membrane-spanning segments 1 and 7 are glycosylated, confirming their luminal location. The region comprising membrane-spanning segments 2–6 shows sequence resemblance to putative sterol-sensing domains in three other proteins: 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), the Niemann-Pick C1 protein, and the morphogen receptor Patched. The orientation of the eight membrane-spanning segments in SCAP is consistent with the model proposed for HMG-CoA reductase (Olender, E. H., and Simoni, R. D. (1992) *J. Biol. Chem.* 267, 4223–4235). The membrane-spanning domains of SCAP and HMG-CoA reductase confer sterol sensitivity upon the functional activities of the two molecules. The common membrane topology of the two proteins is consistent with the notion that sterols regulate both proteins by a common mechanism.

about 500 amino acids in length, project into the cytosol. They are joined by a membrane-attachment domain consisting of two membrane-spanning sequences separated by a short hydrophilic loop of 31 amino acids that projects into the ER lumen. The NH₂-terminal domain is a transcription factor of the basic-helix-loop-helix-leucine zipper family (1). The COOH-terminal domain binds SCAP, thereby facilitating the proteolytic release of the NH₂-terminal domain (2, 3).

In cells deprived of cholesterol, the two-step release process begins when a protease clips the SREBPs at Site-1, which is a Leu-Ser bond in the luminal loop (5). This cleavage requires the SREBP-SCAP complex, and it is prevented by a variety of maneuvers that disrupt this complex (2, 3). The cleavage separates the two halves of the SREBPs, but the NH₂-terminal domain remains bound to membranes by virtue of its single membrane-spanning segment. Release is completed when a second protease cleaves the NH₂-terminal segment at a site within the first membrane-spanning domain (1). The responsible enzyme, designated Site-2 protease, was recently identified by molecular cloning as a novel type of polytopic intramembraneous metalloprotease (6).

Cholesterol inhibits its own synthesis by abolishing the cleavage of SREBPs at Site-1 (1). This regulation is thought to be mediated by the interaction of sterols with the membranous domain of SCAP, which contains a segment whose sequence resembles the sterol-sensing domain of HMG-CoA reductase (7) and two other proteins that are thought to interact with sterols, *i.e.* the Niemann-Pick C1 gene product and the developmental protein Patched (8). Sterols do not disrupt the SREBP-SCAP complex, but rather they abolish the ability of this complex to activate the Site-1 protease (2, 3, 7). The latter has not been defined in molecular terms.

Upon entering the nucleus, the NH₂-terminal domains of the SREBPs activate transcription of several genes encoding proteins involved in cholesterol synthesis (*e.g.* HMG-CoA synthase, HMG-CoA reductase, farnesyl diphosphate synthase, squalene synthase, and others), cholesterol uptake (LDL receptor), fatty acid synthesis (acetyl-CoA carboxylase, fatty acid synthase, stearoyl-CoA desaturase), and triglyceride synthesis (glycerol-3-phosphate acyltransferase) (1, 9–13). Sterols down-regulate transcription of each of these genes by blocking proteolytic cleavage of SREBPs (1). This allows cells to maintain a relatively constant membrane composition in the face of widely varying amounts of cholesterol supply and demand.

A full understanding of the mechanism of sterol regulation requires a more complete structural and functional analysis of SCAP, the protein that mediates this control. Studies to date have shown that SCAP comprises two distinct domains: an NH₂-terminal polytopic membranous domain of ~730 amino acids and a COOH-terminal water soluble domain of ~540 amino acids that projects into the cytosol (1, 7). The COOH-terminal domain contains five more or less degenerate copies of a sequence of ~40 residues, which has been called the WD

Sterol regulatory element binding proteins (SREBPs)¹ are membrane-bound transcription factors that activate the synthesis and uptake of cholesterol and fatty acids in animal cells (reviewed in Ref. 1). Their activities are regulated by SREBP cleavage-activating protein (SCAP), a polytopic membrane protein that forms complexes with membrane-bound SREBPs in the endoplasmic reticulum (ER). Formation of this complex is a necessary step in the pathway by which the NH₂-terminal fragments of SREBPs are released proteolytically from the ER membrane, so that they can enter the nucleus to stimulate transcription (2, 3).

The SREBPs are bound to ER membranes in a hairpin fashion (4). The NH₂-terminal and COOH-terminal domains, each

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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; ER, endoplasmic reticulum; HMG, 3-hydroxy-3-methylglutaryl; HSV, herpes simplex virus; LDL, low density lipoprotein; LDLR, LDL receptor; PNGase F, peptide N-glycosidase F; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis.

repeat. Found in a variety of proteins, WD repeat domains are felt to mediate protein-protein interactions (14). This has been shown directly in the crystal structures of heterotrimeric G proteins, whose β -subunits contain seven WD repeats that are required for contacts with the α and γ subunits (15, 16). As one might expect, the COOH-terminal domain of SCAP is the region that interacts with the COOH-terminal regulatory domains of SREBPs (2, 3).

The structure of the membranous NH₂-terminal domain of SCAP has not yet been studied directly. Analysis of the amino acid sequence suggests that this region contains multiple membrane-spanning segments separated by hydrophilic loops of varying length (7). Five of these putative membrane-spanning segments show a sequence resemblance to membrane-spanning segments 2–6 of HMG-CoA reductase (7) whose NH₂-terminal membranous domain (17) has been shown to contain eight membrane-spanning segments (18). Membrane-spanning segments 2–6 of hamster HMG-CoA reductase show 25% sequence identity and 55% similarity to a similar region of SCAP (7). As mentioned above, this same region is shared with the Niemann-Pick C1 gene product and with Patched (8), supporting the concept that this region interacts with sterols. For this reason, the sequence corresponding to membrane-spanning segments 2–6 of HMG-CoA reductase has been called the sterol-sensing domain (1, 7, 8).

In the current studies we have tested the model for the membrane topology of SCAP through protease protection experiments and through an examination of patterns of *N*-linked glycosylation. The data are consistent with the proposed polytopic model.

EXPERIMENTAL PROCEDURES

Materials—We obtained monoclonal antibodies IgG-HSV-TagTM and IgG-T7-TagTM from Novagen, Inc.; monoclonal anti-BiP antibody from StressGene Biotechnologies Corp.; bromelain, soybean trypsin inhibitor, and tosyl-lysine chloromethyl ketone from Sigma-Aldrich, Inc.; trypsin and Triton X-100 from Boehringer Mannheim; Nonidet P-40 and *N*-acetyl-leucinal-leucinal-norleucinal (ALLN) from Calbiochem-Novabiochem Corp.; and peptide *N*-glycosidase F (PNGase F) and restriction enzymes from New England Biolabs, Inc. Other reagents were obtained from sources as described previously (19–21).

Recombinant Plasmids—Expression vectors pTK, pTK-HSV-BP2, and pTK-HSV-SCAP-T7 have been previously described (7, 20). pTK-HSV-SCAP is an expression vector encoding amino acids 2–1276 of wild-type hamster SCAP preceded by an initiator methionine, two tandem copies of the herpes simplex virus (HSV) epitope (QPELAPE-DPED) (22), and two novel amino acids (ID, encoded by the sequence for a *Bsp*DI restriction site). This plasmid is derived from pTK-HSV-SCAP(D443N) (see Ref. 7) by replacement of a *Bst*EII-*Fse*I fragment containing the wild-type codon 443. pTK-HSV-LDLR-SCAP encodes a fusion protein consisting of an initiator methionine, two tandem copies of the HSV epitope, amino acids 811–860 of the human LDL receptor (23), three novel amino acids (TGD, generated by blunt-end ligation of filled-in *Age*I and *Bsp*DI restriction sites), and amino acids 2–1276 of wild-type hamster SCAP. All other expression plasmids used in this study were derived from the above-mentioned vectors by polymerase chain reaction-based, site-directed mutagenesis (24). The structures of all constructs were verified by DNA sequencing of the ligation junctions and all other modified regions.

Transient Transfection of 293 Cells—Monolayers of human embryonic kidney 293 cells were set up on day 0 (5.5×10^5 cells/100-mm dish) and cultured in 8–9% CO₂ at 37 °C in medium A (Dulbecco's modified Eagle's medium containing 100 units/ml penicillin and 100 μ g/ml streptomycin sulfate) supplemented with 10% (v/v) fetal calf serum. On day 2, the cells were transfected with the indicated plasmids using an MBS kit (Stratagene) as described previously (4). Three h after transfection, the cells were switched to fresh medium as indicated in the figure legends, incubated overnight, and harvested on day 3.

Stable Transfection of CHO Cells with Plasmid Encoding Epitope-tagged SCAP—Stock cultures of CHO-7 cells, a clone of CHO-K1 cells (American Type Culture Collection, catalog number CCL-61) adapted to growth in lipoprotein-deficient serum (19), were grown in medium C (a 1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle's

medium containing 100 units/ml penicillin and 100 μ g/ml streptomycin sulfate) supplemented with 5% newborn calf lipoprotein-deficient serum. Colonies of CHO-7 cells stably transfected with a vector expressing epitope-tagged, wild-type hamster SCAP (pTK-HSV-SCAP-T7) were isolated by growth in G418 and 25-hydroxycholesterol as described previously (7). Individual surviving colonies were dilution-cloned, and one clone (LB343-9a-1) was expanded and used for the experiments reported here. Cells were set up for experiments on day 0 (5×10^5 cells/100-mm dish) in medium C supplemented with 5% newborn calf lipoprotein-deficient serum and harvested on day 3.

Isolation of Cell Membranes (Protocol 1)—Each monolayer of 293 or CHO-7 cells was scraped into the culture medium, centrifuged at 1000 *g* for 5 min at 4 °C, resuspended in 1 ml phosphate-buffered saline, and centrifuged again as above. The cell pellet from each dish was resuspended in 0.4 ml sucrose-containing buffer D (10 mM Hepes-KOH at pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 5 mM sodium EDTA, 5 mM sodium EGTA, 250 mM sucrose), passed through a 22-gauge needle 20 times, and centrifuged at 1000 *g* for 5 min at 4 °C. Each supernatant was centrifuged at 1.5×10^4 *g* for 10 min at 4 °C, and the resulting pellet was designated as the membrane fraction.

Isolation of Cell Membranes (Protocol 2)—Each monolayer of CHO-7 cells was scraped into the culture medium, centrifuged, and washed once with phosphate-buffered saline as described above. The cell pellet from each dish was resuspended in 0.2 ml of sucrose-free buffer E (10 mM Hepes-KOH at pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 5 mM sodium EDTA, 5 mM sodium EGTA) and allowed to swell for 10 min at 0 °C on ice. The suspension was passed through a 22-gauge needle 15 times and centrifuged at 1000 \times *g* at 4 °C for 5 min. Each supernatant was subjected to centrifugation in a Beckman TLA 100 rotor at $10^5 \times$ *g* for 45 min, and the resulting pellet was designated as the membrane fraction.

Bromelain Proteolysis—293 or CHO-7 cells were harvested, and cell membranes were prepared by Protocol 1 except that protease inhibitors (see below) were added to buffer D. The membrane pellets were suspended in 0.1 ml buffer F (buffer D supplemented with 0.1 M NaCl, 10 μ g/ml leupeptin, 5 μ g/ml pepstatin A, and 1.9 μ g/ml aprotinin) or in buffer F containing 1% (v/v) Triton X-100 and rocked at 4 °C for 5 min. Varying amounts of bromelain were added in a volume of 2 μ l, and the samples were incubated at 25 °C for 30 min in a final volume of 92 μ l. The reactions were stopped by the addition of 2 μ l of 50 mM tosyl-lysine chloromethyl ketone followed by 25 μ l of buffer G (150 mM Tris-HCl at pH 6.8, 15% (w/v) SDS, 12.5% (v/v) 2-mercaptoethanol, 25% (v/v) glycerol, 0.02% (w/v) bromophenol blue). The samples were then heated at 100 °C for 5 min and subjected to SDS-PAGE.

Trypsin Proteolysis—293 or CHO-7 cells were harvested, and the membrane fraction was prepared by Protocol 1 or 2 as indicated in the figure legends. Membrane pellets from 293 cells were suspended in 0.14 ml of buffer H (buffer D plus 0.1 M NaCl), rocked at 4 °C for 5 min, and divided into 56- μ l aliquots for trypsin treatment. Membrane pellets from CHO-7 cells were suspended in 0.2 ml of buffer I (buffer E plus 0.1 M NaCl). Aliquots (90 μ l) were supplemented with 10 μ l of buffer I or buffer I containing 10% Triton X-100 and rocked at 4 °C for 30 min before trypsin treatment. Varying amounts of trypsin were added in a volume of 2 μ l, and the samples were incubated at 30 °C for the indicated time. The reactions were stopped by the addition of 400 units of soybean trypsin inhibitor (2 μ l) and 25 μ l of buffer G. The samples were then heated at 100 °C for 5 min and subjected to SDS-PAGE.

Sequential Trypsin Proteolysis and Glycosidase Treatment—Membrane fractions from transfected 293 cells were prepared as described in Protocol 1 and suspended in 0.14 ml of buffer H. Aliquots (56 μ l) were treated with 1 μ g of trypsin as described above, and the reactions were stopped by the addition of 2 μ l of soybean trypsin inhibitor (400 units) followed by addition of 10 μ l of solution containing 3.5% SDS and 7% 2-mercaptoethanol. After heating at 100 °C for 10 min, the samples received sequential additions of 7 μ l of 10% (v/v) Nonidet P-40, 7 μ l of 0.5 M sodium phosphate, pH 7.5, and 2 μ l of varying amounts of PNGase F (1 unit = 15.4 IUBMB microunits) as indicated in the figure legends. After incubation at 37 °C for the indicated time, reactions were stopped by the addition of 25 μ l of buffer G. The mixtures were heated at 100 °C for 5 min and subjected to SDS-PAGE.

Immunoblot Analysis—Protein concentration was measured with a BCA kit (Pierce). Monoclonal antibody IgG-9D5 directed against amino acids 540–707 of hamster SCAP was prepared as described previously (2). All other antibodies used in this study were obtained from commercial sources as described above. Gels were calibrated with prestained molecular weight markers (Amersham Pharmacia Biotech or Bio-Rad). After SDS-PAGE, proteins were transferred to Hybond C-extra nitrocellulose sheets (Amersham) and incubated with monoclonal antibodies

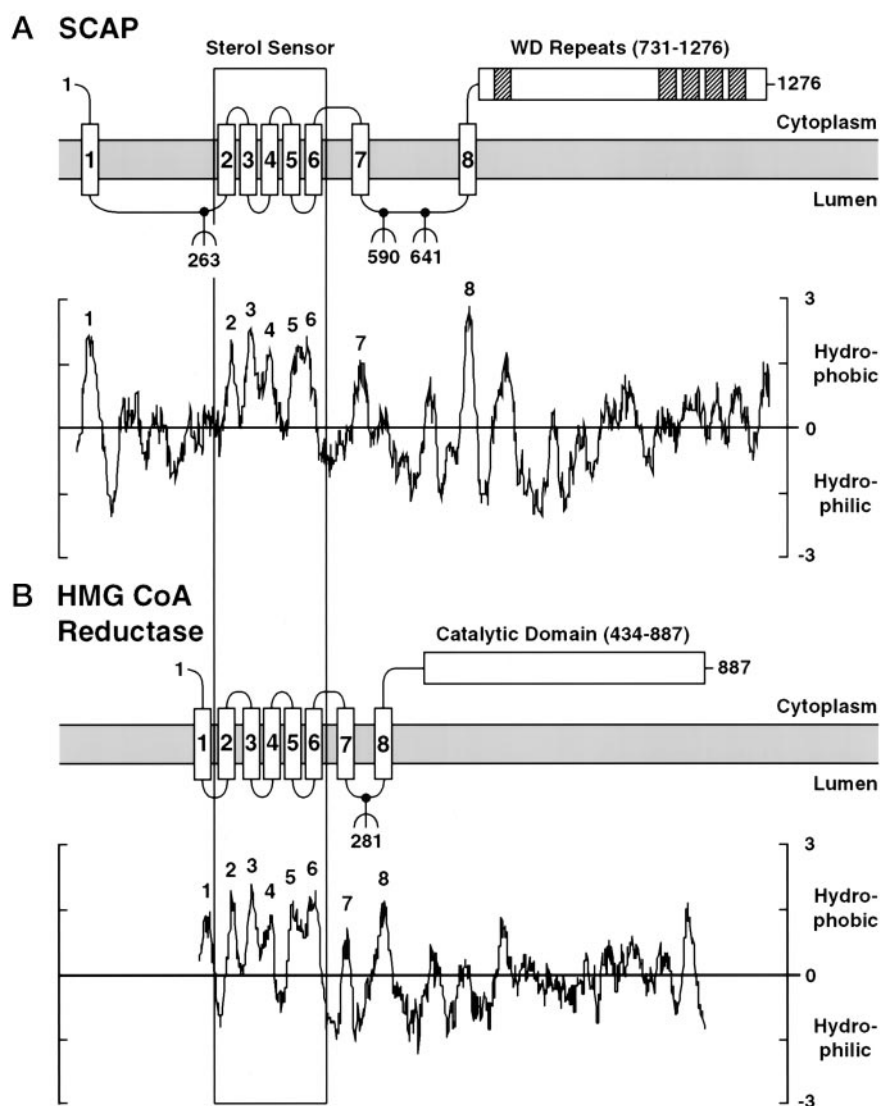


FIG. 1. Predicted membrane topology and hydropathy plot of hamster SCAP (A) and hamster HMG-CoA reductase (B). The topology model for SCAP is based on data in this manuscript; the topology model for HMG-CoA reductase is based on the data of Olender and Simoni (18). The boxed area denotes the region of SCAP and HMG-CoA reductase that shares amino acid sequence identity (7) and is designated as the sterol-sensing domain. The residue-specific hydropathy indices were calculated over a window of 20 residues by the method of Kyte and Doolittle (36) using the Genetics Computer Group Sequence Analysis Software Package, Version 8.0. The numbers below each topology diagram denote the positions of *N*-linked glycosylation sites. The topology models are drawn to scale so that the length of a segment along the horizontal axis is proportional to the number of amino acids in that segment.

at the indicated concentration. Bound antibodies were visualized by chemiluminescence with horseradish peroxidase-conjugated, affinity-purified donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) using the SuperSignal substrate system (Pierce) according to the manufacturer's instructions. Filters were exposed to Reflection™ NEF-496 film (NEN Life Science Products) at room temperature for the indicated time.

RESULTS

Fig. 1A shows a hydropathy plot of the amino acid sequence of SCAP and the predicted membrane topology of the protein. For comparison, we show similar diagrams for HMG-CoA reductase (Fig. 1B) based on the studies by Simoni and co-workers (18, 25). The NH₂-terminal domain of SCAP is predicted to contain eight membrane-spanning regions (numbered 1–8 in the hydropathy plot). The assignment of these predicted segments is based not only upon their relative hydrophobicity but also upon analogy with the eight membrane-spanning segments of HMG-CoA reductase (18, 25). As discussed in the Introduction, putative membrane-spanning regions 2–6 of hamster SCAP show a 25% identity and 55% similarity with membrane-spanning regions 2–6 of hamster HMG-CoA reductase (7). This region of both proteins is called the sterol-sensing domain. The box in Fig. 1 highlights the similar hydropathy plots of the two sterol-sensing domains. Fig. 1A also shows the position of three potential sites of *N*-linked glycosylation (Asn-X-Ser/Thr) that occur in relatively long loops of SCAP that are

postulated to project into the ER lumen. SCAP has two other potential *N*-linked glycosylation sites, but these are located between WD repeats 1 and 2 in the COOH-terminal domain that faces the cytosol (2, 3). It is noteworthy that the single site of *N*-linked glycosylation in HMG-CoA reductase occurs in the loop between membrane-spanning segments 7 and 8 (Fig. 1B). In SCAP, this loop contains two potential *N*-linked glycosylation sites (Fig. 1A).

The model of Fig. 1A predicts that the NH₂ terminus and COOH terminus of SCAP face the cytosol. To test this hypothesis, we prepared an expression vector encoding SCAP with two copies of an epitope tag from the HSV envelope glycoprotein D at the NH₂ terminus and three copies of an epitope from bacteriophage T7 at the COOH terminus (Fig. 2). Previous studies have shown that this epitope-tagged version of SCAP has wild-type activity in its ability to stimulate cleavage of SREBPs (7). The plasmid was introduced into CHO-7 cells by transfection, and a stable cell line expressing the tagged protein was isolated. Membranes were prepared from these cells and digested with increasing amounts of bromelain in the absence or presence of Triton X-100. The proteolytic reaction was stopped, and the proteins were subjected to SDS-PAGE and blotted with an antibody against the COOH-terminal T7 epitope. Membranes from nontransfected CHO-7 cells did not show any reactivity with the anti-T7 antibody (Fig. 2, lanes 1–4). In the transfected

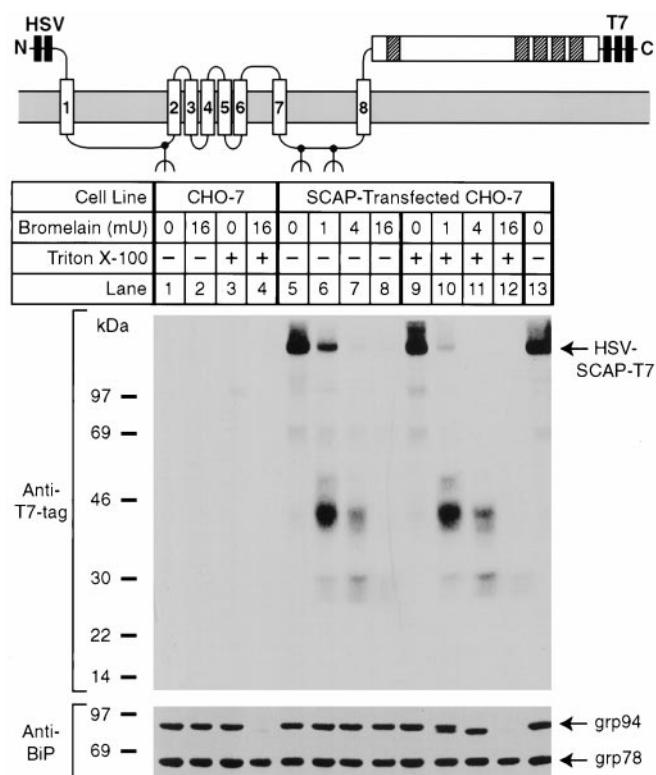


FIG. 2. Membrane orientation of the COOH-terminal domain of SCAP as determined by bromelain proteolysis. The diagram shows a schematic of the fusion protein encoded by pTK-HSV-SCAP-T7, which comprises two copies of the HSV epitope tag, followed by SCAP, followed by three copies of the T7 epitope tag. CHO-7 cells (lanes 1–4) or CHO-7 cells stably transfected with pTK-HSV-SCAP-T7 (lanes 5–13) were harvested, and membrane fractions were prepared by Protocol 1 as described under “Experimental Procedures.” Aliquots of membranes (81 μ l) were treated in a final volume of 92 μ l with the indicated amount of bromelain in the absence or presence of 1% Triton X-100 as indicated. After incubation for 30 min at 25 $^{\circ}$ C, reactions were stopped, subjected to SDS-PAGE (5–20% gradient gels), and transferred to nitrocellulose. Duplicate filters were blotted with 1 μ g/ml IgG-T7-TagTM antibody (upper panel) or 2 μ g/ml anti-BiP antibody (lower panel) and exposed to film for 3 and 1 s, respectively. *mU*, milliunits.

cells, the epitope-tagged SCAP appeared as a band at \sim 150 kDa (lanes 5, 9, and 13). In the absence of detergent, low concentrations of bromelain produced fragments of \sim 47, 39, and 30 kDa, indicative of three bromelain-sensitive sites within the COOH-terminal domain (lanes 6 and 7). At higher concentrations, bromelain abolished all immunoreactivity (lane 8). The results were identical in the presence of Triton X-100 (lanes 10–12). As a control for the integrity of the membrane vesicles, we blotted duplicate filters with an antibody against grp78 (BiP), a known intraluminal protein of the ER (26). The anti-BiP antibody (directed against the COOH-terminal KSGKDEL sequence (StressGene Biotechnologies Corp.)) also reacts with grp94, another intraluminal KDEL-terminating protein of the ER (26, 27). The grp94 protein was resistant to bromelain in the absence of detergent (lanes 6–8), but it was destroyed in the presence of detergent (lanes 4, 10–12). These results confirm that the membrane vesicles were impermeant to bromelain in the absence of detergent. The grp78 band was not useful for this analysis because it resisted bromelain digestion both in the absence and presence of detergent, indicating that the protein itself is bromelain-resistant under the conditions of these experiments. In earlier studies (4), we found that grp78 also resisted digestion with trypsin in the absence and presence of Triton X-100.

When we blotted the membranes with an antibody against

the HSV epitope tag at the NH₂ terminus of SCAP, the results were not interpretable because a protease-resistant fragment of \sim 44 kDa was detected in the presence as well as absence of Triton X-100. These data are not shown in Fig. 2, but the fragment is shown in another context in Figs. 5 and 6 (see below). We postulated that the inability to destroy the HSV epitope in the presence of Triton X-100 was caused by the short sequence (18 amino acids) between the HSV tags and the first transmembrane segment. To circumvent this problem, we prepared another expression vector in which the HSV epitope was separated from the NH₂ terminus by an artificial spacer sequence of 50 amino acids that was derived from the cytoplasmic COOH-terminal domain of the LDL receptor (Fig. 3). To make certain that this construct was biologically active, we tested its ability to stimulate cleavage of SREBP-2 in the presence of sterols. Human embryonic kidney 293 cells were transiently transfected with a plasmid encoding SREBP-2 with an HSV tag at the NH₂ terminus (HSV-BP2). The cells were incubated in the absence and presence of sterols, and nuclear extracts were subjected to SDS-PAGE and blotted with an antibody to the HSV tag (Fig. 3A). In the absence of sterols, the nuclear extracts contained a protein corresponding to the mature NH₂ terminus of SREBP-2 (lane 3), and this was abolished by sterols (lane 4). Cotransfection of the plasmid encoding HSV-LDLR-SCAP abolished the sterol suppression (lane 5). The effect was similar to the effect that was seen with HSV-SCAP lacking the LDLR spacer (lane 6). As expected, the membrane fraction of these cells contained the HSV-tagged SREBP-2 precursor as well as the HSV-tagged SCAP with (lane 5) or without (lane 6) the LDLR spacer.

Fig. 3B shows the results of a protease protection experiment performed with cells transfected with the cDNA encoding HSV-LDLR-SCAP. Bromelain (10 milliunits) completely destroyed the epitope tag, and the result was similar in the absence or presence of Triton X-100 (lanes 5 and 9). Bromelain destroyed the control protein grp94 only in the presence of detergent, again confirming the intact nature of the membrane vesicles (lanes 6 and 10).

The results of Figs. 2 and 3 indicate that both the COOH-terminal and NH₂-terminal ends of SCAP are oriented toward the cytosol. We next tested the orientation of the long hydrophilic loop that is postulated to lie between membrane-spanning regions 7 and 8 (Fig. 4). For this purpose, we used IgG-9D5, a monoclonal antibody directed against a bacterially expressed fusion protein containing residues 540–707 of SCAP. Membranes from nontransfected CHO-7 cells were incubated with increasing amounts of trypsin in the absence or presence of Triton X-100, and the samples were subjected to SDS-PAGE and blotted with IgG-9D5. In the absence of Triton X-100 we observed a protected fragment of \sim 36 kDa (lane 5). This fragment was not protected in the presence of Triton X-100 (lane 8), indicating that protection results from sequestration within the lumen of the ER. As a control, we showed that grp94 was destroyed by trypsin only in the presence of Triton X-100 (bottom panel, lanes 7 and 8). The size of the protected fragment corresponded to the size expected if trypsin had cut SCAP in the exposed hydrophilic loop preceding membrane-spanning region 7 and the segment that follows membrane-spanning region 8, thus indicating that these segments face the cytosol (Fig. 4).

The next series of experiments was designed to determine whether N-linked glycosylation occurs at any or all of the three potential luminal sites shown in Fig. 1. Treatment of the intact SCAP protein with PNGase F led to a small increase in mobility on gel shift assays, but the results could not be dissected further because of the large size of SCAP and the relatively

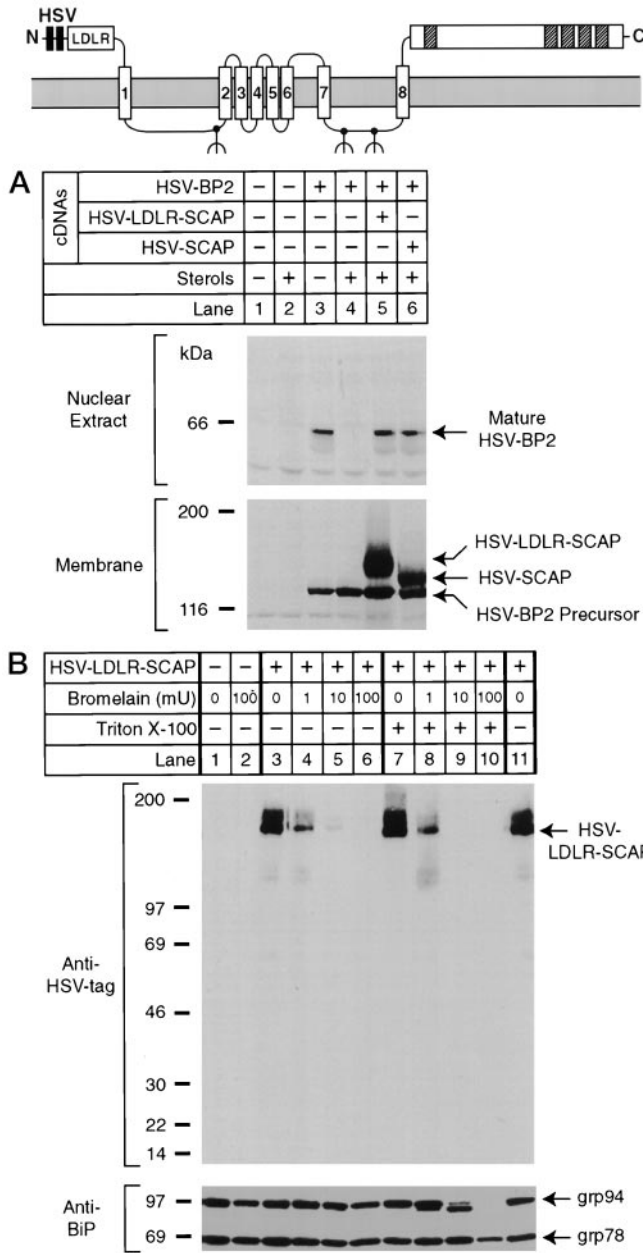


FIG. 3. Membrane orientation of the NH₂ terminus of SCAP as determined by bromelain proteolysis. The diagram shows a schematic of the fusion protein encoded by pTK-HSV-LDLR-SCAP, which comprises two copies of the HSV epitope tag followed by the COOH-terminal 50 amino acids of the human LDL receptor followed by amino acids 2–1276 of SCAP. *A*, 293 cells were transfected with 10 μ g of control pTK plasmid (*lanes 1 and 2*) or 9 μ g pTK-HSV-BP2 (*lanes 3–6*) plus 1 μ g of pTK-HSV-LDLR-SCAP or pTK-HSV-SCAP as indicated. The total amount of DNA in *lanes 3 and 4* was adjusted to 10 μ g by the addition of 1 μ g of pTK. Three h after transfection, cells were switched to medium B (medium A containing 10% lipoprotein-deficient serum, 50 μ M compactin, 50 μ M sodium mevalonate, 0.2% (v/v) ethanol) in the absence (– sterols) or presence (+ sterols) of 1 μ g/ml 25-hydroxycholesterol plus 10 μ g/ml cholesterol as indicated. After incubation for 19 h at 37 °C, 25 μ g/ml ALLN was added directly to the medium for an additional 4 h, after which the cells were harvested and fractionated as described previously (20, 37). Aliquots of nuclear extracts (100 μ g, *upper panel*) and membranes (100 μ g, *lower panel*) were subjected to 7% SDS-PAGE, transferred to nitrocellulose, and blotted with 0.5 μ g/ml IgG-HSV-Tag™ antibody. Filters were exposed to film for 1 s. *B*, 293 cells were transfected with 10 μ g of either control plasmid (pTK, *lanes 1 and 2*) or pTK-HSV-LDLR-SCAP (*lanes 3–11*). Three h after transfection, the cells were switched to medium A supplemented with 10% fetal calf serum. After incubation for 24 h, cells were harvested, and membrane fractions were prepared by Protocol 1 as described under “Experimental Procedures.” Aliquots of membranes (30 μ l) were treated in a

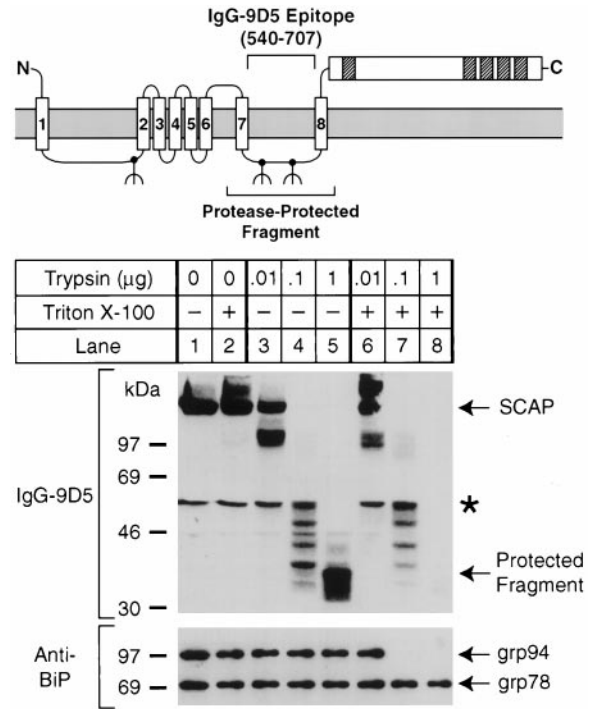


FIG. 4. Membrane orientation of amino acids 540–707 of SCAP as determined by trypsin proteolysis. The diagram shows a schematic of SCAP, denoting the location of amino acids 540–707, which constitute the epitope recognized by monoclonal antibody IgG-9D5. Membrane fractions from nontransfected CHO-7 cells were prepared by Protocol 2 as described under “Experimental Procedures,” and aliquots were treated with the indicated amount of trypsin in a final volume of 102 μ l in the absence or presence of 1% Triton X-100 as indicated. After incubation for 15 min at 30 °C, the reactions were stopped, subjected to SDS-PAGE (5–15% gradient gels), and transferred to nitrocellulose. Duplicate filters were blotted with 10 μ g/ml IgG-9D5 (*upper panel*) or 2 μ g/ml anti-BiP antibody (*lower panel*) and exposed to film for 30 s. The asterisk denotes a cross-reacting protein of unknown identity.

small shift upon deglycosylation. To circumvent this problem, we took advantage of the small sizes of the protease-protected fragments of SCAP. 293 cells were transfected with the plasmid encoding SCAP with an HSV tag at the NH₂ terminus (Fig. 5). Membranes were digested with trypsin in the absence of detergent. The reaction was stopped by the addition of soybean trypsin inhibitor, after which we added increasing amounts of PNGase F, which should remove all N-linked carbohydrates. After incubation for 1 h, the samples were subjected to SDS-PAGE and blotted with an antibody against the NH₂-terminal HSV tag (*panel A*) or IgG-9D5 directed against the loop between membrane-spanning regions 7 and 8 (*panel B*). After trypsin treatment, the anti-HSV antibody revealed a protease-protected fragment of ~44 kDa (*panel A*). As discussed above, the ability to visualize this fragment depended on the observation that the NH₂-terminal HSV tag was resistant to trypsin digestion. When the protected fragment was treated with PNGase F, its apparent molecular mass was reduced in a single step to ~42 kDa, suggesting that this fragment contained a single N-linked sugar. As shown in *panel B*, the IgG-9D5 antibody visualized a protected fragment of ~36 kDa. Increasing amounts of PNGase F reduced the size of this fragment to ~32

final volume of 92 μ l with the indicated amount of bromelain in the absence or presence of 1% Triton X-100 as indicated. After incubation for 30 min at 25 °C, reactions were stopped, subjected to SDS-PAGE (5–20% gradient gels), and transferred to nitrocellulose. Duplicate filters were blotted with either 0.5 μ g/ml IgG-HSV-Tag™ antibody (*upper panel*) or 2 μ g/ml anti-BiP antibody (*lower panel*) and exposed to film for 5 and 15 s, respectively.

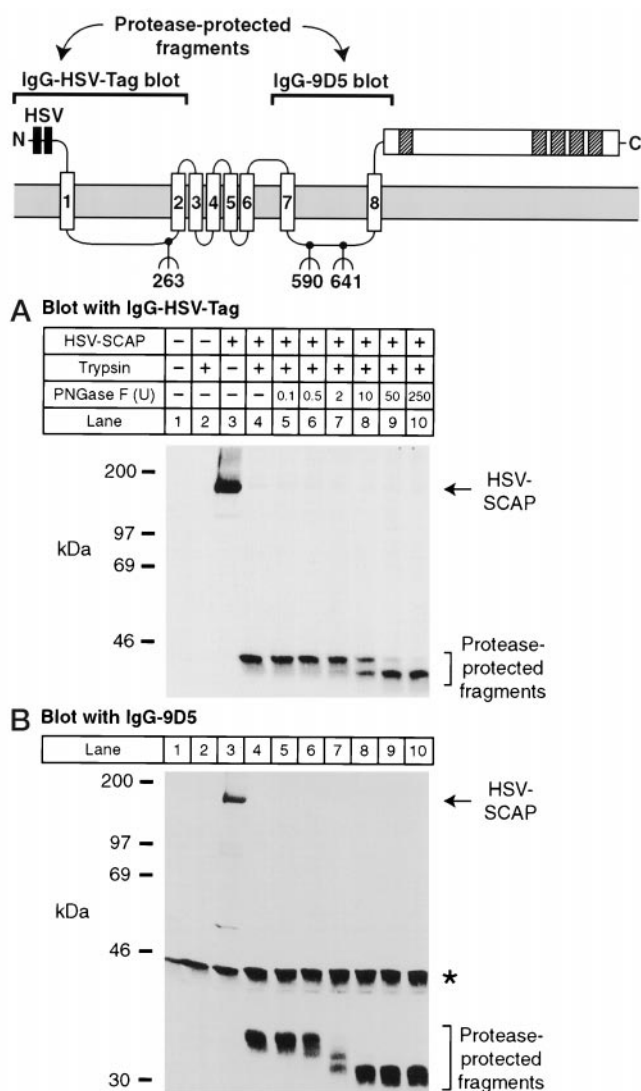


FIG. 5. Glycosylation of SCAP at all three N-linked sites in the luminal loops. The diagram shows a schematic of the fusion protein encoded by pTK-HSV-SCAP, illustrating the approximate positions of protease-resistant fragments recognized by monoclonal antibodies IgG-HSV-TagTM (A) and IgG-9D5 (B), respectively. Numbers below the diagram indicate sites of N-linked glycosylation. 293 cells were transfected with 10 μ g of control pTK plasmid (lanes 1 and 2) or 5 μ g of pTK-HSV-SCAP plus 5 μ g pTK (lanes 3–10). Three h after transfection, the cells were switched to medium A supplemented with 10% fetal calf serum. After incubation for 19 h at 37 °C, membrane fractions were prepared by Protocol 1 as described under “Experimental Procedures.” Aliquots of the membranes were suspended in buffer without detergent and incubated for 30 min at 25 °C in a final volume of 58 μ l in the absence or presence of 1 μ g of trypsin as indicated. Proteolysis was stopped, the samples were incubated for 1 h at 37 °C in a final volume of 86 μ l in the absence or presence of the indicated amount of PNGase F, subjected to 10% SDS-PAGE, and transferred to nitrocellulose. The filter was blotted with 0.5 μ g/ml IgG-HSV-TagTM and exposed to film for 8 s (upper panel). The same filter was stripped by incubation in 64 mM Tris-chloride, pH 6.7, 2% SDS, and 0.1 M 2-mercaptoethanol for 30 min at 50 °C, reblotted with 10 μ g/ml IgG-9D5, and exposed to film for 45 s (lower panel). The asterisk denotes a cross-reacting protein of unknown identity.

kDa. An intermediate fragment of ~34 kDa was also seen. This result is consistent with the presence of two N-linked sugars in the protected fragment visualized by IgG-9D5.

To test for glycosylation at each of the three sites individually, we prepared a series of plasmids encoding HSV-tagged versions of SCAP in which one or two of the asparagine residues were changed to glutamine, thereby abrogating glycosy-

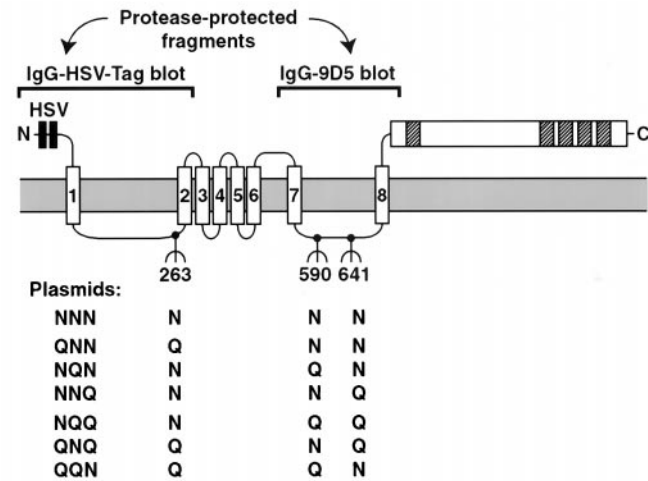
lation at that site. Unfortunately, we were unable to study a protein in which all three asparagines had been replaced. The triple-replacement construct failed to produce a protein that could be visualized with any of our antibodies (data not shown). Apparently, at least one carbohydrate chain is required for proper folding and/or stability of SCAP. The results with the glycosylation site mutations are shown in Fig. 6. For simplicity, we designate the three glycosylation sites according to the amino acid residue that initiates the glycosylation signal sequence (N for the wild-type asparagine and Q for the mutant glutamine). The wild-type protein is therefore NNN, where the first N refers to the site in the first luminal loop, and the second and third Ns refer to the sites in the loop between membrane spans 7 and 8. Fig. 6A shows the results of immunoblots with the anti-HSV antibody, which visualizes only the protease-protected, first luminal loop. As shown above, when all three glycosylation sites were intact, the mobility of this fragment increased after treatment with PNGase F (lanes 4 and 5). When the first asparagine was replaced with glutamine (QNN), the mobility of this fragment increased, and there was no further effect of PNGase F (lanes 6 and 7), indicating that the glycosylated sequence was indeed the Asn-X-Ser in the first luminal loop. This site continued to be glycosylated when we abolished either or both of the glycosylation sites in the loop between membrane spans 7 and 8 (lanes 8–13).

Fig. 6B shows the results when the same proteolytic digest was blotted with an antibody against the 9D5 epitope in luminal loop 7–8. As shown above, with the wild-type sequences (NNN) the mobility of this fragment increased after PNGase F treatment (lanes 4 and 5). Elimination of the first glycosylation site had no effect (QNN, lanes 6 and 7). When either the second or third glycosylation site was eliminated (NQN and NNQ, respectively), the mobility of the protected fragment was faster than that of the wild-type protein, but it continued to be increased by PNGase F treatment (lanes 8–11). Replacement of both asparagines in this loop (NQQ) increased the mobility of the fragment so that it equaled that of the deglycosylated protein, and there was no further effect of PNGase F (lanes 12 and 13).

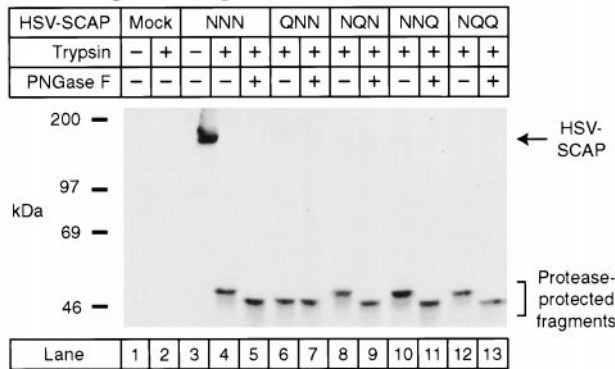
The experiment of Fig. 7 was designed to determine whether the glycosylation-deficient mutants of SCAP retain the ability to stimulate proteolytic cleavage of SREBPs. 293 cells were transfected with a cDNA encoding SREBP-2 with an NH₂-terminal HSV epitope tag. Nuclear extracts were subjected to SDS-PAGE and blotted with anti-HSV to visualize the mature NH₂-terminal fragment of SREBP-2. This fragment was visualized when the cells were incubated in the absence of sterols (lane 3), and it was abolished by sterols (lane 4). We next tested the reversal of this suppression when the cells were cotransfected with cDNAs encoding wild-type or mutant versions of SCAP. Wild-type SCAP alleviated sterol suppression (lane 11). A similar result was obtained with each of the single or double glycosylation mutants (lanes 5–10). These findings indicate that a single carbohydrate chain is sufficient for SCAP activity and that this chain can be located in either the first or last luminal loop. The bottom panel shows the membrane fraction blotted with IgG-9D5 against SCAP. Each of the transfected SCAP cDNAs produced a similar amount of protein.

DISCUSSION

The current data support the model shown in Fig. 1A for the membrane orientation of SCAP. The protease protection experiments demonstrate that the NH₂ terminus and COOH terminus of SCAP are sensitive to proteases and hence, they face the cytosol. The long loops that follow the first and seventh membrane-spanning segments are protease-resistant and glycosylated, indicating that they face the lumen. These experiments



A Blot with IgG-HSV-Tag



B Blot with IgG-9D5

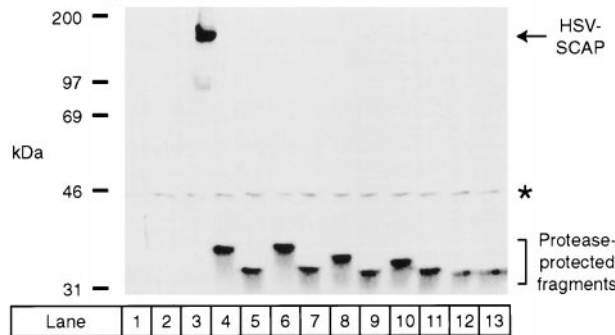


FIG. 6. Glycosylation of SCAP mutants harboring point mutations at individual N-linked sites. The diagram shows a schematic of the fusion protein encoded by pTK-HSV-SCAP, illustrating the approximate positions of protease-resistant fragments recognized by monoclonal antibodies IgG-HSV-Tag and IgG-9D5. Numbers below the diagram denote sites of N-linked glycosylation. The table summarizes the glycosylation sites in wild-type and mutant SCAP proteins encoded by transfected plasmids used for this experiment. 293 cells were transfected with the following plasmids: lanes 1 and 2, 10 μ g of control pTK plasmid; lanes 3–5, 7 μ g of pTK-HSV-SCAP; and lanes 6–13, 7 μ g of mutant versions of pTK-HSV-SCAP with asparagine-to-glutamine mutations introduced at the indicated N-linked glycosylation sites. The total amount of DNA in lanes 3–13 was adjusted to 10 μ g by the addition of 3 μ g of pTK. Three h after transfection, the cells were switched to medium A supplemented with 10% fetal calf serum. After incubation for 24 h at 37 $^{\circ}$ C, membrane fractions were prepared and treated as described in the legend to Fig. 5. Aliquots of the membranes were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4–13) of 1 μ g of trypsin. Proteolysis was stopped, and the samples were incubated for 12 h in the absence or presence of 500 units PNGase F as indicated. The samples were then subjected to SDS-PAGE (5–15% gradient gel), transferred to nitrocellulose, and blotted with 0.5 μ g/ml IgG-HSV-Tag (A). The same filter was stripped as described in Fig. 5 and reblotted with 10 μ g/ml IgG-9D5 (B). Filters were exposed to film for 2 s (A) and 8 s (B), respectively. The asterisk denotes a cross-reacting protein of unknown identity.

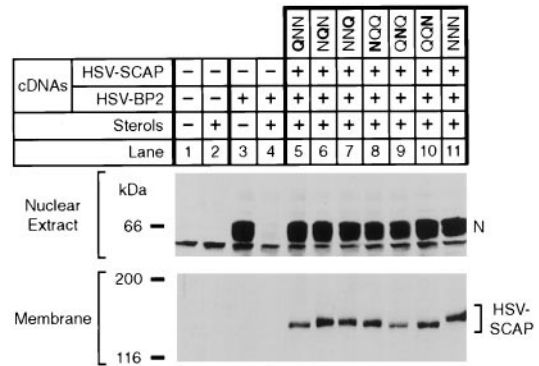


FIG. 7. Comparison of SREBP cleavage-stimulating activity of wild-type and glycosylation-deficient forms of SCAP. 293 cells were transfected with 10 μ g of control plasmid (pTK; lanes 1 and 2), 9 μ g of pTK-HSV-BP2 (lanes 3–11), and 1 μ g of the indicated forms of pTK-HSV-SCAP (see Fig. 6; lanes 5–11). The total amount of DNA in lanes 3 and 4 was adjusted to 10 μ g by the addition of 1 μ g of pTK. Three h after transfection, cells were switched to medium B (medium A containing 10% lipoprotein-deficient serum, 50 μ M compactin, 50 μ M sodium mevalonate, 0.2% ethanol) in the absence (- sterols) or presence (+ sterols) of 1 μ g/ml 25-hydroxycholesterol plus 10 μ g/ml cholesterol as indicated. After incubation for 22 h at 37 $^{\circ}$ C, 25 μ g/ml ALLN was added directly to the medium for an additional 4 h, after which cell fractionation was carried out as described previously (20, 37). Aliquots of nuclear extracts (50 μ g, upper panel) and membranes (100 μ g, lower panel) were subjected to 7% SDS-PAGE, transferred to nitrocellulose, and blotted with 0.5 μ g/ml IgG-HSV-TagTM antibody (upper panel) or 10 μ g/ml IgG-9D5 (lower panel). Filters were exposed to film for 15 s. N denotes the nuclear mature form of HSV epitope-tagged SREBP-2.

define the orientation of membrane-spanning segments 1 and 8. The hydrophobicity plot indicates that membrane-spanning segments 2–6 are closely spaced and are separated from each other by very short hydrophilic loops ranging in size from ~5 to 10 amino acids. The orientation of these segments has been inferred from a comparison with the sterol-sensing domain in HMG-CoA reductase (Fig. 1 and Refs. 7 and 18).

The model of Fig. 1 indicates a remarkably similar overall organization for SCAP and HMG-CoA reductase. Like SCAP, HMG-CoA reductase has a polytopic membrane attachment domain with eight membrane-spanning segments followed by a long COOH-terminal domain that projects into the cytosol. In HMG-CoA reductase, as in SCAP, the loop that follows membrane-spanning segment 7 is relatively long, but it is much longer in SCAP than in HMG-CoA reductase. The last loop of HMG-CoA reductase, like the one in SCAP, is glycosylated (17, 18). The orientation of membrane-spanning segments 2–6 in HMG-CoA reductase has been determined by Simoni and co-workers (18, 25) on the basis of deletion mutagenesis, epitope tagging, glycosylation site mapping, and the preparation of fusion proteins. The results indicated that the NH₂ terminus of membrane-spanning segment 2 faces the lumen, and the COOH terminus of membrane-spanning segment 6 faces the cytosol. The model of SCAP in Fig. 1A is consistent with this orientation.

In both SCAP and HMG-CoA reductase, the long cytosolic COOH-terminal domains are the functional units, and the polytopic membrane-spanning segments confer sensitivity to regulation by sterols. The COOH-terminal domain of HMG-CoA reductase contains all of the catalytic activity of the enzyme (17). Indeed, this domain alone can confer sterol-independent growth when transfected into HMG-CoA reductase-deficient mutant CHO cells (28). The COOH-terminal domain of SCAP is also the active component. It forms a complex with SREBPs and thereby accelerates their proteolytic cleavage and activation (2, 3).

Although SCAP and HMG-CoA reductase are both regulated

by sterols, they are regulated in different ways. Sterols regulate HMG-CoA reductase by accelerating the degradation of the protein by a proteolytic system that is inhibited by ALLN (29) and thus may represent proteasomes (30). Sterols regulate SCAP by interfering with its ability to stimulate the proteolytic cleavage of SREBPs, but they do not appear to accelerate the degradation of SCAP, and ALLN does not affect the ability of SCAP to confer sterol regulation upon SREBP cleavage (7).

We have previously described a point mutation in SCAP that markedly reduces its sensitivity to inhibition by sterols (7, 31). This mutation (D443N) replaces a residue that lies at the COOH terminus of membrane-spanning segment 6 and hence is within the region that is shared with HMG-CoA reductase. Aspartate 443 itself is not conserved in hamster HMG-CoA reductase where the equivalent position is occupied by valine (7). However, the residue corresponding to aspartate 443 is conserved in the Niemann-Pick C1 protein and in Patched (8).

Niemann-Pick type C1 cells have a block in the transport of lipoprotein-derived cholesterol from the lysosome to the ER (32, 33). The responsible protein has at least 12 putative membrane-spanning regions (8), and it therefore differs fundamentally in organization from SCAP and HMG-CoA reductase. Nevertheless, the cholesterol transport function of this protein is likely to be mediated, at least in part, by the conserved sterol-sensing domain.

Patched is a polytopic membrane protein that serves as one component of the receptor for Hedgehog proteins, a family of signaling morphogens that induce cellular differentiation in several organs during development (34). Hedgehog proteins are the only known proteins that contain covalently bound cholesterol molecules (35). Hedgehog proteins are synthesized as precursors that undergo an autocatalytic cleavage reaction that proceeds through an internal thioester intermediate that is cleaved by cholesterol. In the final product cholesterol remains covalently bound to the COOH terminus of the active fragment of Hedgehog. This active fragment binds to Patched, even in the absence of the cholesterol adduct (34, 35). Nevertheless, it seems likely that the covalently bound cholesterol may modulate this interaction by binding to the sterol-sensing domain of Patched.

A crucial unresolved question is whether sterols regulate SCAP and the other sterol-sensing proteins by interacting directly with the sterol-sensing domain or whether sterols interact with another protein that binds to this domain. This is a difficult question to answer for technical reasons, because of the insolubility of sterols and their tendency to partition into the membranes or detergents that surround the sterol-sensing membrane proteins. The task may be made somewhat easier now that the orientation of this domain in at least two of these proteins (HMG-CoA reductase and SCAP) is known.

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