Membrane Topology of S2P, a Protein Required for Intramembranous Cleavage of Sterol Regulatory Element-binding Proteins*

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In sterol-depleted mammalian cells, a two-step proteolytic process releases the NH2-terminal domains of sterol regulatory element-binding proteins (SREBPs) from membranes of the endoplasmic reticulum (ER). These domains translocate into the nucleus, where they activate genes of cholesterol and fatty acid biosynthesis. The SREBPs are oriented in the membrane in a hairpin fashion, with the NH2- and COOH-terminal domains facing the cytosol and a single hydrophilic loop projecting into the lumen. The first cleavage occurs at Site-1 within the ER lumen to generate an intermediate that is subsequently released from the membrane by cleavage at Site-2, which lies within the first transmembrane domain. A membrane protein, designated S2P, a putative zinc metalloprotease, is required for this cleavage. Here, we use protease protection and glycosylation site mapping to define the topology of S2P in ER membranes. Both the NH2- and COOH termini of S2P face the cytosol. Most of S2P is hydrophobic and appears to be buried in the membrane. All three of the long hydrophilic sequences of S2P can be glycosylated, indicating that they all project into the lumen. The HEIGH sequence of S2P, which contains two potential zinc-coordinating residues, is contained within a long hydrophobic segment. Aspartic acid 467, located ~300 residues away from the HEIGH sequence, appears to provide the third coordinating residue for the active site zinc. The hydrophobicity of these sequences suggests that the active site of S2P is located within the membrane in an ideal position to cleave its target, a Leu-Cys bond in the first transmembrane helix of SREBPs.

Animal cells maintain cholesterol homeostasis by regulating proteolysis of sterol regulatory element-binding proteins-1 and -2 (SREBP-1a and -1c) (1). Release of the NH2-terminal domain of SREBP must be released from the membrane so that it can enter the nucleus. This release has been studied most extensively for one of the SREBPs, namely, SREBP-2. However, the mechanism appears to be similar for the other SREBPs (SREBP-1a and -1c) (1). Release of the NH2-terminal domain is accomplished by a two-step proteolytic event that is regulated by sterols (3). In sterol-depleted mammalian cells, this proteolysis is initiated by the Site-1 protease (S1P), which cleaves human SREBP-2 between the Leu522-Ser523 bond in the sequence RSVL S (4). This cleavage requires formation of a complex between SREBP and SCAP, a polytopic membrane protein of the ER, and it is prevented when this complex is disrupted (5, 6).

Cleavage at Site-1 breaks the covalent bond between the two transmembrane domains of SREBP-2, but both parts of the protein remain attached to the membrane. Cleavage by Site-2 protease is necessary to release the transcriptionally active NH2 terminus of SREBP-2 into the cytosol, from which it rapidly translocates into the nucleus. The released fragment terminates at leucine 484, suggesting that Site-2 protease cleaves between this residue and cysteine 485 (7). This bond is believed to lie within a membrane-spanning helix. Cleavage is dependent on an upstream tetrapeptide sequence, DRSR, which precedes the transmembrane sequence (3, 8).

Cleavage at Site-1 is tightly regulated by sterols. When cultured cells are loaded with sterols, either by incubation with plasma low density lipoprotein (LDL) or a mixture of cholesterol and 25-hydroxycholesterol, this cleavage is abolished (9). Cleavage at Site-2 is not directly regulated by sterols, but it cannot occur without prior cleavage at Site-1 (3). Therefore, it occurs only in sterol-depleted cells.

Upon entering the nucleus, the NH2-terminal domains of the SREBPs activate transcription of genes encoding multiple enzymes of the cholesterol biosynthetic pathway (e.g. 3-hydroxy-3-methylglutaryl-CoA synthase, 3-hydroxy-3-methylglutaryl-CoA reductase, farnesyl diphosphate synthase, squalene synthase, and lanosterol synthase) and the fatty acid biosynthetic pathway (e.g. fatty acid synthase, acetyl-CoA carboxylase, and stearoyl-CoA desaturase) (reviewed in Refs. 1, 10, and 11). They also directly activate transcription of the gene encoding the LDL receptor, which supplies cells with cholesterol and fatty acids from external sources. Sterols down-regulate the

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† The abbreviations used are: SREBP, sterol regulatory element-binding protein; CMV, cytomegalovirus; ER, endoplasmic reticulum; HSV, herpes simplex virus; LDL, low density lipoprotein; LDLR, LDL receptor; PNGase F, peptide N-glycosidase F; SRE-1, sterol regulatory element-1; SIP, Site-1 protease; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary.

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transcription of these genes by interfering with the proteolysis of SREBPs. This feedback regulation allows cells to maintain a steady membrane composition in a changing environment (1).

Our laboratory recently isolated a cDNA encoding a protein that is a candidate for the Site-2 protease. We named this protein S2P. The cDNA was identified by complementation cloning (12) in M19 cells, which are a mutant line of CHO cells that cannot cleave SREBPα at Site-2 (3). As a result of this defect, the M19 cells are unable to transcribe genes encoding cholesterol biosynthetic enzymes or the LDL receptor, and they are therefore cholesterol auxotrophs (3, 12, 13). M19 cells fail to produce an mRNA encoding S2P, owing to a genomic rearrangement that disrupts the S2P gene (12). When transfected into M19 cells, the cDNA encoding S2P restores Site-2 cleavage of SREBPs and relieves the growth requirement for cholesterol.

The human S2P cDNA encodes a protein of 519 amino acids that contains a consensus metalloprotease-zinc-binding site, HEIGH (12). Replacement of either of the two histidines or the glutamic acid destroys the ability of the S2P cDNA to restore SREBP cleavage in M19 cells (12). These findings are compatible with the hypothesis that S2P is indeed a metalloprotease (14), but so far, multiple attempts to demonstrate protease activity of isolated S2P in vitro have failed. S2P differs from known metalloproteases in its extensive hydrophobicity, which suggests the existence of multiple membrane-embedded domains. The protein also contains a stretch of 23 contiguous serine residues beginning at amino acid 114 and a cysteine-rich region containing 10 cysteines (residues 285–377). These two domains constitute the most hydrophilic parts of the protein.

A full understanding of the mechanism of the S2P cleavage reaction requires a more complete structural and functional analysis of S2P. In the current study, we propose a model for the membrane topology of S2P and test it through examination of patterns of N-linked glycosylation and protease susceptibility.

**EXPERIMENTAL PROCEDURES**

**Materials**—CHO-K1 cells are a clone of CHO-K1 cells selected for growth in lipoprotein-deficient serum (15). M19 cells are a mutant line of CHO-K1 cells auxotrophic for cholesterol and un saturated fatty acids (13), owing to a deletion in the S2P gene (12). We obtained monoclonal antibody IgG-HSV-Tag™ from Novagen, Inc.; monoclonal anti-BiP antibody from StressGen Biotechnologies Corp.; trypsin (Cat. No. 109827) from sources as described previously (8, 9, 15).

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**Recombinant Plasmids**—All vectors expressing S2P or S2P mutants were driven by the cytomegalovirus (CMV) promoter-enhancer in the pcDNA3 vector (Invitrogen). Expression vectors pCMV-HSV-S2P and pCMV-Myc-S2P have been previously described (12). pCMV-HSV-LDLR-S2P is an expression vector encoding a fusion protein consisting of an initiator methionine, two tandem copies of the HSV epitope (QPELAPEDPED) (16), amino acids 811–860 of the human LDL receptor (17), three novel amino acids (TG), and two tandem copies of the c-Myc epitope (EQKLISETEDLN) (18), two novel amino acids (ID) encoded by the sequence for the Cys-rich region containing 10 cysteines (residues 285–377). These two domains constitute the most hydrophilic parts of the protein.

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relatively hydrophilic with several charged amino acids. This is constitutive a membrane-spanning helix. Residues 21–70 are hydrophobic segments that are postulated to lie within the membrane.

Asterisks denote two potential sites of N-linked glycosylation: Asn-337 and Asn-508. On day 0, 293 cells were set up for experiments as described under “Experimental Procedures.” A, on day 2, the cells were transfected with 5 μg/dish of either the control vector pcDNA3 (lane 1) or the indicated plasmid (lanes 2–6). Three h after transfection, the cells were switched to fresh medium A supplemented with 10% fetal calf serum. After incubation for 20 h at 37 °C, membrane fractions were prepared as described under “Experimental Procedures.” Aliquots of the membrane fractions (30 μg of protein) were subjected to SDS-PAGE and immunoblotted with 0.5 μg/ml of IgG-HSV-Tag. The filter was exposed to film for 15 s at room temperature. B, aliquots of the membrane fractions were treated at 37 °C for 1 h as described under “Experimental Procedures” with one of the following glycosidases: lane 1, none; lane 2, 0.0038 IU of PNGase F; lane 3, 0.25 IU of endoglycosidase H; lane 4, 0.83 IU of neuraminidase. Aliquots of each reaction (30 μg of protein) were subjected to SDS-PAGE and immunoblotted with 0.5 μg/ml of IgG-HSV-Tag. The filter was exposed to film for 30 s at room temperature.

**Results**

Model for Membrane Topology of S2P—The top panel of Fig. 1 shows a hydropathy plot of S2P, and the middle panel shows the predicted membrane topology of the protein. The bottom panel shows the amino acid sequence with hydrophobic regions indicated by shading. The three hydrophilic segments that are believed to reside in the ER lumen are designated A–C. Hydrophobic segments that are postulated to lie within the membrane are denoted by dashed lines in the middle panel.

The sequence of S2P contains an uneven distribution of the charged amino acids lysine, arginine, glutamate, and aspartate (indicated by asterisks in the bottom panel of Fig. 1). Amino acids 1–20 are extremely hydrophobic and are predicted to constitute a membrane-spanning helix. Residues 21–70 are relatively hydrophilic with several charged amino acids. This is designated region A. Residues 71–107 are again hydrophobic and are predicted to insert into the membrane. These residues are followed by the second long hydrophobic segment, designated region B, which extends from residues 108–141. Region B contains 26 serines, 23 of which are contiguous. Residue 141 begins a very long hydrophobic sequence that ends at residue 258. This sequence is interrupted by a short hydrophilic sequence that begins with the HEIGH sequence at position 171 and extends to the arginine at 185. Four out of 15 residues in this segment are charged. The long sequence between residues 258 and 446, designated region C, contains numerous charged and polar amino acids. This sequence also contains multiple cysteines. The sequence between residues 447 and 476 contains only one charged amino acid and is predicted to dip into the membrane. It is followed by a short hydrophilic segment, residues 477–491, the location of which could not be assigned from these studies (see below). The protein terminates with another hydrophobic sequence extending from residues 492 to 519. The sole charged residue in this segment is the COOH-terminal arginine.

N-Linked Glycosylation of S2P—S2P contains two sequences that conform to the Asn-X-Ser/Thr consensus for N-linked glycosylation (asparagines 337 and 508) as indicated in Fig. 2. Asparagine 337 is in the cysteine-rich hydrophilic loop (region C), and asparagine 508 is in the COOH-terminal hydrophobic sequence. In order to determine whether N-linked glycosyl-
tion occurs at either of these sites, we created mutants of S2P in which one or both of the asparagines were replaced with glutamine residues. We prepared expression vectors encoding wild-type or mutant S2P with two copies of an epitope tag from an HSV protein at the NH2 terminus. The plasmids were transiently transfected into human embryonic kidney 293 cells, membrane fractions were prepared, and these membranes were subjected to SDS-PAGE and blotted with an antibody against the HSV epitope. Membranes from mock-transfected 293 cells showed no immunoreactivity with the anti-HSV antibody (Fig. 2A, lane 1). Epitope-tagged wild-type S2P migrated on the gel as a smear of bands between 45 and 49 kDa (lanes 2 and 6). When asparagine 337 was replaced with glutamine, only the smallest band (~45 kDa) was observed (lane 3). When asparagine 508 was substituted with glutamine, the mobility was indistinguishable from wild-type (lane 4). Double mutants in which both asparagines were replaced with glutamines behaved in the same fashion as the single N337Q mutant (lane 5). These results indicate that the asparagine at position 337, but not the one at position 508, is glycosylated. We therefore infer that region C projects into the ER lumen.

When extracts containing HSV-tagged wild-type S2P were treated with PNGase F, the mobility of the protein increased on SDS-PAGE, confirming that the protein contained an N-linked carbohydrate chain (Fig. 2B, lane 2). The same change was seen after treatment with endoglycosidase H (lane 3), but not with neuraminidase (lane 4). These data indicate that the carbohydrate chain of S2P has not been modified by α-mannosidase II, which is found in the cis-Golgi compartment, or by sialyltransferase, which is found in the trans-Golgi (20).

Glycosylation Is Not Essential for S2P Function—The experiments shown in Fig. 3 were designed to determine whether the function of S2P requires its glycosylation. For this purpose, we used M19 cells, which lack S2P, owing to a deletion in the S2P gene (12). In Fig. 3A, M19 cells were transfected with an expression plasmid containing either no cDNA insert or a cDNA encoding the indicated HSV-tagged wild-type or mutant S2P. We cotransfected a reporter plasmid encoding luciferase driven by a promoter that contains three copies of sterol regulatory element-1 (SRE-1). After incubation in the presence or absence of sterols, the cells were harvested. The measured values for SRE-1 driven luciferase activity were normalized for transfection efficiency by measurement of β-galactosidase activity generated by a cotransfected plasmid encoding β-galactosidase driven by a sterol-independent promoter. In the absence of cotransfected S2P, luciferase activity was low in the absence or presence of sterols (empty vector). When wild-type pCMV-HSV-S2P was transfected, luciferase activity was high in the absence of sterols (closed bars) and was reduced to basal levels in their presence (open bars). As presented in detail earlier (12) these data indicate that wild-type S2P restores cleavage of SREBPs at Site-2, an event that occurs only after the sterol-regulated cleavage at Site-1. The single and double glycosylation site mutants of HSV-S2P also led to enhanced sterol-regulated transcription of the reporter gene, although this enhancement was somewhat reduced when compared with that produced by wild-type S2P. All proteins encoded by the transfected mutant constructs were expressed at levels similar to that of the wild-type (data not shown).

To further test the activity of the nonglycosylated versions of S2P, we compared the ability of the cDNAs encoding wild-type and glycosylation-negative S2P to restore growth of M19 cells in cholesterol-depleted medium (Fig. 3B). When transfected with empty vector, the M19 cells grew in medium supplemented with cholesterol, mevalonate, and oleate, but they failed to grow in the absence of these ingredients, owing to the deficiency of nuclear SREBPs. When the M19 cells were transfected with cDNAs encoding either wild-type or glycosylation-defective S2P, the cells grew in the absence or presence of these supplements. These results parallel the transcription data in Fig. 3A and indicate that S2P does not require glycosylation in order to restore Site-2 cleavage in M19 cells.

Insertion of Glycosylation Sites into S2P—To determine the membrane orientation of the hydrophilic regions of S2P, we transfected 293 cells with expression plasmids encoding epitope-tagged mutant versions of S2P that contained additional Asn-Ser/Thr sequences inserted into these regions (Fig. 4). The cells were harvested, and N-linked glycosylation was detected by treatment with PNGase F followed by SDS-PAGE and immunoblotting with an antibody against the epitope tag. As described above, wild-type HSV-S2P migrated as a cluster of bands between 45 and 49 kDa (Fig. 4, lane 2). Treatment with PNGase F increased the mobility of the pro-
in the absence or presence of 0.0038 IU of PNGase F as described in the

37 °C, membrane fractions were prepared and incubated for 1 h at 37 °C

supplemented with 10% fetal calf serum. After incubation for 20 h at

4–11, mutant versions of pCMV-HSV-S2P described in the diagram.

The diagram shows the sites of insertion of a pair of

glycosylation site sequences in four different regions of HSV-S2P (de-

noted A–D). N337 denotes the endogenous N-linked glycosylation site of

S2P. 293 cells were transfected with 5 μg/dish of the following plasmids:

lane 1, control pcDNA3; lanes 2 and 3, wild-type pCMV-HSV-S2P; lanes

4–11, mutant versions of pCMV-HSV-S2P described in the diagram.

Three h after transfection, the cells were switched to fresh medium A

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legend to Fig. 2. Each sample (30 μg of protein) was then subjected to

SDS-PAGE and immunoblot analysis with 0.5 mg/ml of IgG-HSV-Tag.

Filters were exposed to film for 15 s.

Membrane orientation of the hydrophilic loops of S2P

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legend to Fig. 2. Each sample (30 μg of protein) was then subjected to

SDS-PAGE and immunoblot analysis with 0.5 mg/ml of IgG-HSV-Tag.

Filters were exposed to film for 15 s.
To confirm that the Myc-S2P-LDLR-HSV and HSV-LDLR-S2P proteins insert correctly into the membrane, we created versions with additional sites for \( N \)-linked glycosylation. These proteins, designated Myc-S2P-LDLR-HSV-GLYCO and HSV-LDLR-S2P-GLYCO, are analogous to the proteins that were used in the protease protection experiment, except that they contain a pair of \( N \)-linked glycosylation sites in the serine-rich hydrophilic loop (region B). We transfected 293 cells with the four constructs containing the 50-amino acid LDLR spacer, subjected membrane fractions to SDS-PAGE, and blotted with an antibody against the HSV tag (Fig. 5C). Proteins that contained additional sites of \( N \)-linked glycosylation (lanes 2 and 4) migrated on the gel more slowly than their counterparts without these sites (lanes 1 and 3), indicating that Myc-S2P-LDLR-HSV-GLYCO and HSV-LDLR-S2P-GLYCO had become glycosylated at the newly introduced sites. These results indicate that introduction of the 50-amino acid LDLR spacer at either the NH\(_2\) or COOH terminus does not alter the overall topology of S2P.

Candidate for Third Zinc-coordinating Residue—Most HEXXH-containing zinc metalloproteases have a third residue that acts together with the two histidines to coordinate the active site zinc (14, 23). This residue can be histidine, tyrosine, glutamate, or aspartate. In the prototypic example, thermolysin, the third coordinating residue is a glutamate that is located on a helix that lies adjacent to the helix that contains the HELTH sequence (23). In an attempt to locate the postulated third zinc ligand in S2P, we created point mutations in several

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**Fig. 5. Membrane orientation of NH\(_2\) and COOH termini of S2P as determined by trypsin proteolysis.** A. A schematic illustration of the fusion proteins used in this experiment. The fusion protein Myc-S2P-LDLR-HSV consists sequentially of two copies of the Myc epitope tag, amino acids 2–519 of S2P, the COOH-terminal 50 amino acids of the human LDL receptor, and two copies of the HSV epitope tag. The fusion protein Myc-S2P-LDLR-HSV-Glyco is an engineered version of Myc-S2P-LDLR-HSV in which two \( N \)-linked glycosylation sites have been inserted immediately after serine 121 and serine 131 in the S2P sequence. The fusion protein HSV-LDLR-S2P consists sequentially of two copies of the HSV epitope tag, the COOH-terminal 50 amino acids of the human LDL receptor, and amino acids 2–519 of S2P. The fusion protein HSV-LDLR-S2P-Glyco is an engineered version of HSV-LDLR-S2P in which two \( N \)-linked glycosylation sites have been inserted immediately after serine 121 and serine 131 in the S2P sequence. B, 293 cells were transfected with 5 \( \mu \)g/dish of control plasmid pcDNA3 (lanes 1 and 2), pCMV-Myc-S2P-LDLR-HSV (lanes 3–10), or pCMV-HSV-LDLR-S2P (lanes 11–18). Three h after transfection, cells were switched to fresh medium A supplemented with 10% fetal calf serum. After incubation at 37 °C for 20 h, cells were harvested, and membrane fractions were prepared as described under “Experimental Procedures.” Aliquots of membranes were treated with the indicated amount of trypsin 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, and transferred to nitrocellulose. Duplicate filters were immunoblotted with either 0.5 \( \mu \)g/ml of IgG-HSV-Tag antibody (upper panel) or 2 \( \mu \)g/ml anti-BiP antibody (lower panel) and exposed to film for 5 s (lanes 1–10, upper panel), 45 s (lanes 11–18, upper panel), or 10 s (lanes 1–18, lower panel). C, 293 cells were transfected with 5 \( \mu \)g/dish of the indicated plasmid. Three h after transfection, cells were switched to fresh medium A supplemented with 10% fetal calf serum. After incubation for 20 h, cells were harvested, and membrane fractions were prepared as described under “Experimental Procedures.” Aliquots of the membrane fractions (50 \( \mu \)g of protein) were subjected to SDS-PAGE and immunoblotted with 0.5 \( \mu \)g/ml of IgG-HSV-Tag. Lanes 1 and 2 were exposed to film for 30 s, and lanes 3–5 were exposed for 5 s.
of the candidate residues that are conserved in the eukaryotic S2P-related proteins (12). The mutated cDNAs were transfected into M19 cells, and the restoration of Site-2 protease activity was assayed with the SRE-luciferase reporter gene described above. As shown in Fig. 6A, all of the tested proteins restored S2P activity, with the single exception of the D467N mutant, which lost all activity. Immunoblotting experiments showed that the D467N protein was expressed at levels similar to wild-type, and it had a similar migration on SDS-PAGE, indicating that it inserted properly into the ER and had become glycosylated (data not shown). In a growth rescue experiment, all of the mutant S2P plasmids except D467N were able to rescue the growth of M19 cells in the absence of cholesterol (Fig. 6B).

**DISCUSSION**

The current results support the model presented in Fig. 1 for the membrane topology of S2P. The protease protection assays indicate that the NH$_2$ and COOH termini of S2P face the cytosol. Potential N-linked glycosylation sites introduced into regions A, B, and C become glycosylated in vivo, suggesting that all three hydrophilic loops project into the lumen. Most of the remaining regions of S2P are hydrophobic and are likely to be associated with the membrane as indicated by the dashed lines in Fig. 1. The only predicted luminal sequence that could not be located unambiguously is the segment of 14 amino acids that immediately precedes the COOH-terminal membrane-spanning sequence. When a potential N-linked glycosylation site was inserted into this loop, it failed to become glycosylated (data not shown). We believe that this hydrophilic loop is too short to be accessible to glycosyltransferases. We infer that this loop is in the lumen because it immediately precedes the final membrane-spanning sequence, the other end of which is in the cytosol, as revealed by the protease protection experiments.

Sequences resembling S2P were originally noted in the genomes of diverse species, including human, hamster, *Drosophila melanogaster*, and the Archaea *Sulfolobus solfataricus* (12). Each of these proteins contains the HEXXXH motif, emphasizing the importance of this putative zinc binding site. The length and relative positions of the hydrophilic sequences are conserved, and in each case, the HEXXXH sequence occurs in the context of an otherwise hydrophobic segment. The hydrophilic sequences differ, however. The hamster protein contains fewer contiguous serines in the polyserine stretch (17 versus 23 in the human). This number is further reduced in *Drosophila* to 4. Remarkably, the *Sulfolobus* sequence lacks both the serine-rich region (region B) and the cysteine-rich region (region C) (12). The conservation of sequence from Archaea to humans suggests that the ancestor of S2P played a housekeeping role. Whether mammalian S2P retains any general housekeeping role or whether it is devoted only to SREBP cleavage is unknown. If S2P does have other roles, they are not required for growth of hamster cells in tissue culture. Hamster M19 cells, which lack S2P, grow normally in culture as long as they are supplied with the end products of the SREBP pathway.

Recently, Lewis and Thomas (24) used the Kyte-Doolittle method and other algorithms to identify conserved potential membrane-spanning regions in human and hamster S2P and related sequences from other species that were obtained from data base searches. The computer model predicted that the hydrophobic regions are organized into six helices, each of which completely spans the membrane. In order to place the serine-rich and cysteine-rich loops in the ER lumen, the computer model specified that the NH$_2$ and COOH termini must both face the lumen (24). This prediction is not fulfilled in the current experiments. Indeed, the protease protection studies indicated that the NH$_2$ and COOH termini of S2P both face the cytosol. This finding can be explained if some of the hydrophobic sequences dip into the membrane but do not cross it, as shown in Fig. 1. One of these dipping sequences is located between luminal loops A and B, both of which can be glycosylated. If the hydrophobic sequence between them crossed the membrane, then only one of these sequences could be in the lumen. The other dipping sequence is located between loop C and the final membrane-spanning sequence at the COOH terminus. If this sequence spanned the membrane, it would reverse the orientation of the COOH-terminal hydrophobic sequence, which would then place the COOH terminus of the protein in the lumen. Such a topology would be inconsistent with the protease protection data in Fig. 5B.

Among the 26 S2P-related sequences compiled by Lewis and
Thomas (24) is a protein from Bacillus subtilis that they designated SP4G. This appears to be the same protein that is more generally called SpoIVFB (25, 26). Lewis and Thomas pointed out that this protein is known to be required for the proteolytic processing of Pro-oK, a transcription factor that is activated in mother cells that are producing endosporides in response to nutrient deprivation. The cleavage site in Pro-oK lies within a hydrophobic segment that may be inserted into a membrane, and expression studies suggest that SpoIVFB is the protease that cleaves this sequence (26). Thus S2P and SpoIVFB are both independently postulated to be proteases that cleave hydrophobic segments of proteins. The other 25 S2P-related proteins have no assigned function (24).

The classic example of an HEXXH-containing zinc endopeptidase is the enzyme thermolysin from Bacillus thermoproteolyticus. The crystal structure of this enzyme was originally deduced in 1972 (27), and it has been studied extensively ever since (23). The catalytic zinc atom is located in the interface between two alpha helices. One helix contains the HEXXH sequence, and the other contains a glutamic acid that also coordinates with the zinc atom. Among the residues that are conserved in all 26 S2P-related proteins is the sequence ΦDG, where Φ is almost always a hydrophobic residue, most frequently leucine. In S2P, the aspartate of the LDG sequence is found at residue 467. In the current studies, we changed this aspartate to asparagine and observed that the protein lost the ability to restore Site-2 cleavage of SREBPs in M19 cells (Fig. 6). This finding, together with the observed conservation of this sequence, strongly suggests that aspartate 467 is the third residue that coordinates the zinc at the active site of S2P. Aspartate 467 is contained within the hydrophobic segment that separates the cysteine-rich region (loop C) and the final transmembrane helix (Fig. 1). If this residue does contribute to the active site, then this hydrophobic segment (indicated by dashed lines in Fig. 1) must be adjacent to the hydrophobic helix that contains the HEXXH sequence. Further experiments will be required to test this hypothesis more fully.2

With regard to the function of S2P in SREBP processing, two crucial questions remain to be answered. First, is S2P indeed a protease? Second, does it directly cleave the Leu-Cys bond in the transmembrane region of SREBPs? The evidence that S2P is a protease is based upon the retention of the HEXXH sequence and the ΦDG sequence in species as distant from humans as Sulfolobus and the demonstration that the two histidines and the glutamate, as well as aspartate 467, are all required in order for S2P to carry out its function in facilitating the cleavage of SREBPs (12). The second question is more difficult to answer from available data. Even if S2P is a protease, it is possible that its role is to activate another protease that in turn cuts the Leu-Cys bond in SREBPs. Alternatively, it is possible that S2P cleaves SREBPs at an intermediate site that is between leucine 523 (the site of cleavage by S1P) and leucine 484 (the final cleavage that releases the NH2-terminal fragment of SREBPs from membranes). If so, this intermediate cleavage must be required in order for a third protease to cleave the Leu484Cys485 bond. These questions cannot be resolved until conditions are found in which to assay the proteolytic activity of S2P in vitro.

Further progress requires the establishment of an in vitro assay of S2P function in cleaving SREBP or in activating another protease to do so. This is not a straightforward task in view of the complexity of the natural substrate. This substrate is an intramembranous peptide bond that is not exposed until a prior cleavage has taken place in a short intralumenal loop, i.e. at Site-1. Recreating this substrate in vitro has so far proven difficult, and the difficulty is not lessened by the fact that S2P is a highly hydrophobic protein that requires detergent for solubilization.

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*The aspartic acid of the conserved LDG sequence in S2P is also required for the proteolytic activity of SpoIVFB from B. subtilis. When this residue was changed to an asparagine, the processing of Pro-oK was blocked and sporulation was reduced by 3–4 orders of magnitude (D. Rudner and R. Losick, personal communication).*