Autocatalytic Processing of Site-1 Protease Removes Propeptide and Permits Cleavage of Sterol Regulatory Element-binding Proteins*

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Site-1 protease (S1P) is a subtilisin-related protease that cleaves sterol regulatory element-binding proteins (SREBPs) in the endoplasmic reticulum lumen, thereby initiating a process by which the transcriptionally active NH₂-terminal fragments of SREBPs are released from membranes. In the current experiments, we transfected cDNAs encoding epitope-tagged hamster S1P into HEK-293 cells or mutant hamster cells that lack S1P. Protease protection assays showed that the bulk of S1P is in the endoplasmic reticulum lumen, anchored by a COOH-terminal membrane-spanning segment. Cleavage of the NH₂-terminal signal sequence of S1P generates S1P-A (amino acids 23–1052), which is inactive. The protein is self-activated by an intramolecular cleavage at Site-B, generating S1P-B (amino acids 138–1052) and liberating a 115-amino acid propeptide that is secreted intact into the medium. The sequence at Site-B is RSLK, which differs from the RSVL sequence at the cleavage site in SREBP-2. S1P-B is further cleaved at an internal RRL sequence to yield S1P-C (amino acids 187–1052). Mutational analysis suggests that S1P-B and S1P-C are both active in cleaving SREBP-2 in a fashion that requires SREBP cleavage-activating protein. The activity of S1P-C may be short-lived because it appears to be transported to the Golgi, a site at which SREBP-2 cleavage may not normally occur. These data provide the initial description of the processing of a subtilisin-related protease that controls the level of sterol-binding protein in blood and cells. In an accompanying paper (Cheng, D., Espenshade, P. J., Slaughter, C. A., Jaen, J. C., Brown, M. S., and Goldstein, J. L. (1999), J. Biol. Chem., 274, 22805–22812), we develop an in vitro assay to characterize the activity of purified recombinant S1P.

Cholesterol metabolism in animal cells is controlled by the sterol-regulated proteolysis of membrane-bound transcription factors called sterol regulatory element-binding proteins (SREBPs) (1, 1). The sterol-regulated reaction is catalyzed by Site-1 protease (S1P), a membrane-bound subtilisin-related serine protease that cleaves SREBPs in a hydrophilic loop that projects into the lumen of the endoplasmic reticulum (ER) and other organelles (2). An understanding of the mechanism of regulation of S1P is essential if we are to understand how animals control the cholesterol content of cells and blood.

SREBPs are a family of three proteins, each of ~1150 amino acids in length. After synthesis, each SREBP is inserted into the membranes of the ER and nuclear envelope in a hairpin orientation (1). The NH₂-terminal segment of ~480 amino acids is a transcription factor of the basic helix-loop-helix-leucine zipper family that projects into the cytoplasm. The middle segment of ~80 amino acids consists of two membrane-spanning helices separated by a luminal hydrophilic loop of ~30 amino acids. The COOH-terminal segment of ~590 amino acids extends into the cytoplasm, where it forms a complex with the COOH-terminal segment of a membrane-bound regulatory protein designated SREBP-cleavage activating protein (SCAP). The SREBP/SCAP complex is the true substrate for S1P; disruption of this complex in intact cells abrogates the proteolytic reaction (2, 3).

S1P initiates the processing of SREBPs by cleaving at a site in the middle of the luminal loop. This reaction has been studied most extensively for human SREBP-2. S1P cleaves this protein between the leucine and serine of the sequence RSVL (4). The specificity of recognition has been studied by transfecting cDNAs encoding mutant forms of SREBP-2 into cultured cells. Cleavage of SREBP-2 absolutely requires arginine or lysine at the P4 position. Although the full range of residues at the P1 position was not studied, cleavage was markedly reduced when the leucine at P1 was replaced by alanine or by the closely related valine. The serine at the P1 position and the serine and valine at the P3 and P2 positions could be replaced by alanine with no effect on cleavage.

Cleavage by S1P separates the SREBP into two fragments, each of which has a single membrane-spanning sequence. This separation allows a second protease, designated Site-2 protease (S2P), to cleave the NH₂-terminal fragment at a position within its membrane-spanning sequence (5, 6). This cleavage releases the NH₂-terminal segment of SREBP, allowing it to enter the nucleus, where it binds to enhancers and activates transcription of genes encoding the low density lipoprotein (LDL) receptor and multiple enzymes of cholesterol and fatty acid biosynthesis (1). When sterols accumulate in cells, the cleavage of SREBPs by S1P is abolished; SREBPs remain attached to membranes; and transcription of the target genes declines. This regulation is mediated by the sterol-sensing domain of SCAP (1, 7). The mechanism by which SCAP stimulates the Site-1 cleavage reaction and the mechanism by which sterols block this stimulation is unknown.

S1P was identified by expression cloning in SRD-12B cells, a mutant line of Chinese hamster ovary cells that is unable to synthesize cholesterol or to take up LDL. The defect in these

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The abbreviations used are: SREBP, sterol regulatory element-binding protein; CMV, cytomegalovirus; endo H, endoglycosidase H; ER, endoplasmic reticulum; HEK-293 cells, human embryonic kidney 293 cells; PAGE, polyacrylamide gel electrophoresis; S1P, Site-1 protease; S2P, Site-2 protease; SCAP, SREBP cleavage-activating protein; TK, thymidine kinase; kb, kilobase(s).
cells was traced to a block in the Site-1 cleavage reaction (8), and this defect was corrected when the cells were transfected with pools of cDNAs obtained from wild-type hamster cells (2). The complementing cDNA was isolated and shown to encode S1P. Subsequent analysis showed that the SRD-12B cells harbor mutations in the S1P gene that abrogate the production of any S1P mRNA. A DNA database search revealed that the sequence of human S1P had been deduced previously as a part of a random cDNA sequencing project in Japan (9). The hamster and human proteins are 97% identical (2).

Hamster S1P is a large polypeptide of 1052 amino acids. The NH2-terminal 22 amino acids are hydrophobic and constitute a classic signal sequence. This is followed by a domain of ~290 amino acids that distinguishes S1P as a subtilisin-related protease (2). By analogy with other family members, it was possible to identify the classic catalytic triad as aspartate 218, histidine 249, and serine 414 (2, 10, 11). Replacement of any one of these amino acids rendered S1P unable to complement the defect in SRD-12B cells, confirming that these residues are required for catalytic activity (2). Following the subtilisin homology domain, there is a stretch of ~520 amino acids that has no resemblance to other proteins. This is followed by a hydrophobic sequence of 25 amino acids that appears to be a classic membrane-spanning anchor and a short COOH-terminal tail of 30 amino acids that is predicted to extend into the cytosol. This cytosolic sequence is unusual because it is extremely rich in proline and basic residues. Cell fractionation studies confirmed that S1P is membrane-bound (2).

The superfamily of subtilisin-related proteases contains more than 200 members that are found in all living organisms (10). The mammalian members of this family are the proforms and the prohormone convertases, which act in the Golgi apparatus or secretory vesicles to process peptides prior to secretion or movement to the cell surface (12). All of these enzymes cleave after basic residues, often after dibasic sequences. They also require a basic residue at the P4 position. The classic recognition sequence is RXR/K/R, where X stands for any amino acid (12). S1P differs from the mammalian members of the subtilisin family because it cleaves after a hydrophobic amino acid, leucine. In this respect, it resembles some of the bacterial subtilases, typified by Savinase from Bacillus lantus (13).

Seidah et al. (14) recently reported the cloning of a human subtilisin-related cDNA, called SKI-1, which is identical to S1P. These authors identified SKI-1 in a polymerase chain reaction-based screen for subtilisin-like proteins that cleave after nonbasic residues. In transfection experiments, they demonstrated that SKI-1 cleaved the precursor form of a secreted peptide, brain-derived neurotrophic factor from a recombinant virus, and human proteins are 97% identical (2).

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All of the known subtilisin family members are synthesized as inactive precursors that are activated by a cleavage reaction that releases an NH2-terminal propeptide (10, 12). Seidah et al. (14) provided evidence that SKI-1/S1P undergoes a processing reaction based on an increase in mobility on SDS-PAGE, but the site of cleavage was not identified.

In the current studies, we show that S1P is indeed synthesized as an inactive membrane-bound precursor and that it undergoes several cleavages that produce active enzymes. The most important cleavage occurs after the sequence RSLK and appears to be autocatalytic. In an accompanying paper, we produce a truncated, secreted form of activated S1P and describe its catalytic properties (17).
plasmid containing a 0.8-kb HindIII fragment of human SREBP-2 ligated to a 3.0-kb fragment of pBluescript KS—linearized by digestion with HindIII. Using this plasmid, we mutated amino acids 519–522 of SREBP-2 (RSVL) to either RSLK or RRRL with the oligonucleotide 5′-AGAATGTGCTGTCAGGAGACGAGCGCGGCAG-GACCGCTAGTGGG-3′ or 5′-AGAATGTGCTGTCAGGAGACGAGCGCGGCAG-GACCGCTAGTGGG-3′, respectively. Subsequently, 0.8-kb HindIII fragments of SREBP-2 containing the mutated sequences were ligated into pTK-HSV-BP to generate pTK-HSV-BP(RSLK) and pTK-HSV-BP(RRRL).

In all plasmid constructions, mutations were confirmed by sequencing the relevant regions. pV110 antibody was produced in a nonvaccinated I. RNA gene, which enhances translation of transfected cDNA constructs.

Antibodies—A polyclonal antibody (U1683) against amino acids 1023–1052 of hamster SIP (2) was generated by immunizing rabbits with a mixture of two synthetic peptides corresponding to amino acids 1023–1037 (KAKRSPKRRPRKRAK) and 1038–1052 (POLQTQTHPRKPTSV), each with an NH2-terminal cysteine residue that was conjugated to keyhole limpet hemocyanin using a standard protocol (23). Monoclonal antibodies were obtained commercially as described above.

Cell Culture, Transfection, and Fractionation of Cells—Monolayer cultures of human embryonic kidney 293 cells (HEK-293 cells) were set up for experiments on day 0 (4 x 105 cells/60-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 μg/ml streptomycin) supplemented with 5% fetal calf serum after which the medium was changed every other day. On day 2, the cells were transfected with the indicated plasmids using LipofectAMINE (GIBCO-BRL) according to the manufacturer's instructions as described (2). Three h after transfection, the cells were refed with 5 ml of Medium B supplemented with 5% fetal calf serum. The medium was changed every other day. On day 6, the cells were diluted to Medium B supplemented with 5% newborn calf lipoprotein-deficient serum. On day 7, the cells were collected and washed with ice-cold phosphate-buffered saline. The cell pellet (10–20 x 106) was kept frozen at -80 °C for 40 ml of Buffer A (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% (w/v) SDS, 0.5% (w/v) deoxycholic acid, 150 mM NaCl, 2 mM MgCl2) supplemented with 1 mM Pefabloc®, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, and 5 μg/ml aprotinin. The cell lysate was rocked for 1 h at 4 °C followed by centrifugation at 2 x 105 g for 1 h at 4 °C. The resulting supernatant was incubated with 500 μg of monoclonal antibodies to BiP and the immunoprecipitates from the SIP(1052) cells were each cut from the membrane and subjected to NH2-terminal sequence analysis by automated Edman degradation performed with Procise Model 494 Sequencer from PE Biosystems (Foster City, CA), using standard programming and chemicals as recommended by the manufacturer.

Results

Fig. 1A outlines the structure of the precursor form of SIP and shows the position of three internal cleavage sites that give rise to shortened forms of SIP, designated A, B, and C. To facilitate subsequent studies, we prepared cDNAs that express epitope-tagged versions of SIP under control of the strong CMV promoter/enhancer. These plasmids encode versions of SIP with 3 copies of a Myc epitope tag at the NH2 terminus (Myc-SIP) or the COOH terminus (SIP-Myc). The NH2-terminal Myc tag was inserted immediately after the predicted site of cleavage by signal peptidase so that it would remain with the protein after this cleavage. The COOH-terminal Myc tag was followed by six histidines, which allowed purification of the protein on a nickel column.

Fig. 1B shows a diagram of the SREBP/SCAP complex and the postulated position of membrane-anchored SIP with its active site juxtaposed to the cleavage site in SREBP. To confirm that the bulk of SIP is located in the ER lumen, we performed protease protection assays (Fig. 1, C and D). HEK-293 cells were transfected with cDNAs encoding Myc-SIP (Fig. 1C) or SIP-Myc (Fig. 1D). Intact membrane vesicles were prepared and treated with varying amounts of trypsin either in the absence or presence of the detergent Triton X-100. When the epitope tag was at the NH2 terminus, the tag was not destroyed by trypsin treatment of intact vesicles (Fig. 1C, lanes 3–6), but it was destroyed when the membranes were dissolved with detergent (lanes 9–12), indicating that this end of the protein is luminal. On the other hand, when the Myc tag was at the COOH terminus, it was destroyed by trypsin both in the absence and presence of the detergent (Fig. 1D, lanes 3–6 and 9–12). To confirm that the vesicles were intact, we used an anti-BiP antibody to show that an intraluminal protein, gp94, was protected from trypsin in the absence of detergent, but was destroyed in its presence (Fig. 1B and D, bottom panels). Another protein visualized by the anti-BiP antibody, gp78, was resistant to trypsin even in the presence of detergent, apparently because of intrinsic trypsin resistance of this protein, a phenomenon that has been observed previously (24, 27). These data confirm the membrane orientation of SIP that is shown in Fig. 1B.
To study the processing of S1P, we used SRD-12B cells that lack endogenous S1P. We transfected the cells with pCMV-Myc-S1P and studied the fate of the COOH terminus by immunoblotting with an antibody against the cytoplasmic tail of S1P (Fig. 2A). The immunoblot revealed three bands that we designate S1P-A, -B, and -C (lane 2). To determine whether these protein species result from autoproteolytic processing, we transfected the cells with pCMV-Myc-S1P in which the serine at the active site was mutated to alanine (S414A). In this case, only S1P-A was visualized (Fig. 2A, lane 3), indicating that processing to the B and C forms requires a functional active site and is autocatalytic. Inasmuch as the antibody recognizes the extreme COOH terminus of S1P, the data indicate that S1P-B and S1P-C must have been produced by proteolytic removal of NH2-terminal sequences.

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Fig. 2B shows an experiment designed to analyze the fate of the NH2-termini of propeptide generated during the proteolytic processing of S1P. For this purpose, we used HEK-293 cells, which yield higher levels of protein from transfected cDNAs than do SRD-12B cells. We transfected HEK-293 cells with pCMV-Myc-S1P and subjected aliquots of the culture medium and a 105 g membrane fraction to SDS-PAGE and immunoblotting with an antibody against the NH2-terminal Myc tag. The membranes contained a single immunoreactive band cor-

**Fig. 1. Domain map and membrane topology of S1P as determined by protease protection.** A, structure of Myc-S1P, S1P-Myc, and processed versions of S1P: S1P-A (amino acids 23–1052), S1P-B (amino acids 138–1052), and S1P-C (amino acids 187–1052). The propeptide sequence (Pro) and the subtilisin-like catalytic domain are boxed. The catalytic site serine (Ser414), the c-Myc epitope tags, and the His6 tag are indicated. Amino acid numbers are shown below each diagram. Potential N-linked glycosylation sites are indicated by *asterisks*. The signal sequence (SS) and the transmembrane segment (TM) are labeled. B, proposed membrane topology of S1P and the SREBP/SCAP complex. C and D, determination of membrane topology of S1P by protease protection assays. Each dish of HEK-293 cells was transfected with 2 μg of either pCMV-Myc-S1P (C) or pCMV-S1P-Myc (D) and 2 μg of pcDNA3 as described under "Experimental Procedures." Following transfection, cells were incubated in Medium A supplemented with 5% fetal calf serum. After 18 h, cells were harvested, and membrane fractions (2 × 105 g pellets) were prepared as described under "Experimental Procedures." Aliquots of membranes (corresponding to 0.7 dish of cells) were treated with the indicated amount of trypsin in a volume of 60 μl in the absence or presence of 1% Triton X-100 for 30 min at 30 °C. Reactions were stopped by addition of trypsin inhibitor, and samples were subjected to SDS-PAGE and immunoblot analysis with 2.5 μg/ml anti-Myc monoclonal antibody 9E10 or 2 μg/ml anti-BiP antibody. Filters were probed with anti-Myc and anti-BiP antibodies and exposed to Kodak X-Omat Blue XB-1 film at room temperature for 10 and 1 s, respectively. Molecular mass standards are expressed in kDa.
SREBP Site-1 Protease: Autocatalytic Processing

**Fig. 2. Processing of Myc-S1P in transfected SRD-12B and HEK-293 cells.** A, each dish of SRD-12B cells was transfected with 2 μg of pcDNA3 plus 1 μg of one of the following plasmids: pcDNA3 mock vector (lane 1); pCMV-Myc-S1P (lane 2), or pCMV-Myc-S1P(S414A) (lane 3) as described under “Experimental Procedures.” Cells were incubated in Medium B supplemented with 5% fetal calf serum. After 18 h, cells were harvested, and 10^7 × g membrane fractions were prepared as described under “Experimental Procedures.” Aliquots of membrane (20 μg) were subjected to SDS-PAGE on a 6% gel followed by immunoblot analysis with a 1:250 dilution of rabbit antiserum against the COOH terminus of S1P. The filter was exposed to film for 2 s. B, each dish of HEK-293 cells was transfected with 2 μg of pcDNA3, 1 μg of pVAI, and 1 μg of one of the following plasmids: pcDNA3 mock vector (lanes 1 and 4), pCMV-Myc-S1P (lanes 2 and 5), or pCMV-Myc-S1P(S414A) (lanes 3 and 6) as described under “Experimental Procedures.” Following transfection, cells were incubated in 2 ml of Medium A supplemented with 1% newborn calf lipoprotein-deficient serum. After 25 h, cells were harvested, medium was saved, and 10^5 × g membrane pellets were prepared as described under “Experimental Procedures.” Aliquots of membranes and medium (each corresponding to 0.12 dish of cells) were subjected to SDS-PAGE on a 4–15% gradient gel followed by immunoblot analysis with 2.5 μg/ml anti-Myc 9E10. The filter was exposed to film for 7 s. Pro denotes the processed propeptide of Myc-S1P.

responding to S1P-A (Fig. 2B, lane 2). The medium contained a single band at ~24 kDa (lane 5). This band was not visualized when we transfected the epitope-tagged S414A mutant of S1P (lane 6), indicating that it was generated by autocatalytic cleavage. We believe that this secreted protein fragment represents the NH2-terminal propeptide that is cleaved to generate S1P-B (see below).

S1P contains six potential sites of N-linked glycosylation that are indicated by asterisks in Fig. 1A. When SRD-12B cells were transfected with pCMV-Myc-S1P, the mobility of S1P-A, S1P-B, and S1P-C was increased after treatment with peptide N-glycosidase F, indicating that they all contained N-linked carbohydrate (Fig. 3, lane 3). When the membranes were treated with endo H, the mobility of S1P-A and S1P-B increased, whereas the mobility of S1P-C did not change. As a result, after endo H treatment, S1P-B and S1P-C comigrated on the gel (Fig. 3, lane 4). These findings indicate that the carbohydrate chains of S1P-A and S1P-B remain in the high mannose, endo H-sensitive form characteristic of ER proteins, whereas S1P-C undergoes processing by Golgi mannosidase II.

To identify the sites within S1P at which processing occurs, we transfected SRD-12B cells with pCMV-S1P-Myc, which produces S1P with a Myc tag at the COOH terminus. A permanent line of transfected cells, designated S1P1052), was selected by growth in the absence of cholesterol, and the cells were cultured in bulk in roller bottles. Cell pellets were solubilized, and S1P-Myc proteins were immunoprecipitated with anti-Myc, subjected to SDS-PAGE, and stained with Coomassie Blue (Fig. 4A). As a control, we prepared cell lysates from untransfected SRD-12B cells that were sustained by addition of cholesterol and unsaturated fatty acids to the growth medium. The immunoprecipitates from the S1P1052) cells contained five visible polypeptide bands that were not visualized in the control cells. Each of these bands was excised from a polyvinylidene fluoride blot and sequenced at the NH2-terminal end by Edman degradation. Three of the bands corresponded in size to S1P-A, -B, and -C. The sequences indicated that S1P-A is the product of signal peptidase cleavage at the Gly-Asp bond after residue 22 (see Fig. 4B). S1P-B resulted from cleavage after residue 137 following the sequence RSLK. S1P-C resulted from cleavage after residue 186 following the sequence RRLL. All of these sequences are completely conserved in the human and hamster proteins (Fig. 4B). Sequencing the two remaining bands on the gel revealed that these were calnexin and BiP, two protein chaperones that may form transient complexes with S1P, as they do with many ER proteins (28).

To determine whether the A, B, and C forms of S1P are functional, we prepared cDNAs encoding mutant forms of S1P.
with altered sequences at the B and C cleavage sites. In the Site-B mutant, the RSLK sequence at the B cleavage site was changed to AAAA. In the Site-C mutant, we changed the P4 to P1 positions of the C cleavage site from RRLLR to AAAAA, and we also altered a nearby sequence that might have served as an alternative cleavage site (RRASL at residues 163–167 changed to VAASL) (see Fig. 4B, shaded box). The cDNAs were introduced into SRD-12B cells by transfection, and the processed forms of S1P were subjected to SDS-PAGE and visualized by immunoblotting with the antiserum against the COOH-terminal tail of S1P. As shown in Fig. 5A, the cDNA encoding wild-type S1P yielded bands corresponding to S1P-A, S1P-B, and S1P-C (lane 2), whereas the inactive S414A mutant showed only the S1P-A band (lane 3). Mutation at Site-B eliminated S1P-B, and it markedly reduced S1P-C (lane 4). The Site-C mutant failed to produce S1P-C, but it produced increased amounts of S1P-B (lane 5).

To monitor the cleavage of S1P from the NH2-terminal end, we transfected the wild-type and mutant constructs into HEK-293 cells and studied the secretion of the propeptide by immunoblotting membrane pellets and aliquots of the culture medium with the antibody against the NH2-terminal Myc tag (Fig. 5B). In the membrane pellets, the precursor form of S1P, S1P-A, was present in cells transfected with the wild-type S1P and all of the mutants. The membranes contained increased amounts of a band corresponding to the propeptide when the Site-C mutant was expressed (Fig. 5B, lane 5). The medium contained the propeptide fragment when the cells expressed either wild-type S1P (lane 7) or the Site-C mutant (lane 10) but not when the cells expressed the S414A mutant or the Site-B mutant (lanes 8 and 9).

We interpret the data of Fig. 5 to indicate that the A form of S1P is cleaved initially autocatalytically at Site-B and subsequently at Site-C. If Site-B cleavage is blocked, as in the Site-B mutant, cleavage at Site-C is reduced. On the other hand, inhibiting Site-C cleavage, as in the Site-C mutant, does not interfere with Site-B cleavage. Because Site-B cleavage occurs first, a single Myc-tagged propeptide is secreted. This protein co-migrates on SDS-PAGE with a recombinant protein that corresponds to amino acids 23–137 of S1P (data not shown).

We used the Site-B and -C mutants to determine whether S1P-A, -B, or -C is active in cleaving SREBP-2 (Fig. 6). For this purpose, we transfected SRD-12B cells with a cDNA encoding a version of human SREBP-2 with an HSV epitope tag at the NH2 terminus. We cotransfected cDNAs encoding wild-type S1P or one of the mutants. Cell membrane pellets and nuclear extracts were analyzed by immunoblotting with the antiserum against the COOH-terminal tail of S1P.

Fig. 4. NH2-terminal sequences of proteins precipitated by anti-Myc in cells expressing Myc-tagged S1P. A, roller bottles of SRD-12B cells and S1P(1052) cells expressing pCMV-S1P-Myc were lysed in Buffer A as described in “Experimental Procedures.” The 2 × 106 × g supernatant was immunoprecipitated with monoclonal 9E10 anti-Myc antibody conjugated to agarose as described. Aliquots of the immunoprecipitates (corresponding to one-half of a roller bottle of cells) were solubilized in 2% SDS loading buffer and subjected to 5–20% gradient SDS-PAGE. Half of the gel was stained with Coomassie Blue, and the other half was transferred to a polyvinylidene fluoride membrane and then stained with Coomassie Blue. Bands corresponding to the five Coomassie Blue-stained proteins from the S1P(1052) cells were cut from the membrane and sequenced by automated Edman degradation as described under “Experimental Procedures.” Each band contained about 5 pmol of protein. B, amino acid sequence of hamster and human S1P (residues 1–240). Residue numbers are shown at the right. Identical residues are boxed. The signal sequence is denoted by the solid overbar. The signal peptidase cleavage site is denoted as Site-A (open arrow). The S1P processing sites are designated Site-B and Site-C (closed arrows). The putative recognition sequences at the two processing sites are highlighted in black. Potential processing sites is highlighted in grey. Potential N-linked glycosylation sites are denoted by asterisks.
membrane pellets prepared from the transfected SRD-12B cells in
A with a 1:250 dilution of anti-S1P serum. The filter was exposed to film for 3 min. In the absence of cotransfected S1P, the membranes contained the precursor form of SREBP-2, but there was no nuclear form (Fig 6A, lane 2). The nuclear form appeared when we cotransfected as little as 0.03 μg of wild-type pTK-Myc-S1P (lane 3). As described above, the S414A mutant of S1P was inactive, even when a 10-fold larger amount (0.3 μg) was trans-
A comparison of the data in Fig. 6, the Site-C mutant gave rise only to the A and B forms of S1P. We were not seen with the S414A mutant or the Site-B mutant. Above, wild-type S1P gave rise to the B and C forms, which were not seen with the S414A mutant or the Site-B mutant. The Site-C mutant gave rise only to the A and B forms of S1P. A comparison of the data in Fig. 6, A and B, reveals that wild-type S1P achieved maximal cleavage of SREBP-2 at a concentration at which the expression of S1P was barely detectable (Fig. 6, A and B, compare lane 2). We attribute this observation to the relative insensitivity of the anti-S1P immunoblot and the fact that S1P acts catalytically so that only a small amount of active enzyme is able to cleave all of the accessible SREBP-2.

To directly test the cleavage activity of S1P-A, we repeated the SREBP-2 cleavage assay using a mutant S1P cDNA that contained both the Site-B and Site-C mutations, designated S1P(Site-B + C mutant). SRD-12B cells transfected with the Site B + C mutant (Fig. 6D, lane 4) expressed only S1P-A as revealed by immunoblotting with anti-S1P serum. Thus, mutation at both Site-B and Site-C blocked all detectable processing of S1P-A. In contrast to the results for the individual Site-B and Site-C mutants (Fig. 6A, lanes 11 and 14), cotransfection of SRD-12B cells with SREBP-2 and the Site B + C mutant of S1P resulted in no detectable cleaved nuclear form of SREBP-2 (Fig. 6C, lane 4). These data indicate that S1P-A is an inactive precursor of S1P that requires the removal of the NH₂-terminal propeptide in order to generate the two active forms, S1P-B and S1P-C, that can cleave SREBP-2.

The data shown in Figs. 5 and 6 suggest that S1P-B and S1P-C are produced by autocatalytic cleavage. If this is true, then S1P must be able to recognize sequences corresponding to Site-B and Site-C (RSLK and RRLL, respectively) in addition to the RSVL sequence that is cleaved in SREBP-2. To test this hypothesis, we prepared cDNAs encoding mutant forms of HSV-tagged SREBP-2 with the putative Site-B and Site-C recognition sequences substituted for the native RSVL sequence (Fig. 7). These cDNAs were transfected into SRD-12B cells with or without a cDNA encoding wild-type S1P. Membranes and nuclear extracts were subjected to SDS-PAGE and immunoblotted with anti-HSV. The data revealed that S1P is able to cleave SREBP-2 containing the Site-B sequence (RSLK) (Fig. 7, lane 8) and the Site-C sequence (RRLL) (lane 10), as well as the wild-type sequence (RSVL) (lane 4). As expected, cleavage of the wild-type sequence was abolished when we replaced the arginine at the P4 position with alanine (ASVL) (lane 6). Cleavage of the Site-B and Site-C sequences was also abolished by the arginine to alanine replacement at P4 (data not shown).

The experiment shown in Fig. 8 was designed to reveal whether each S1P molecule cleaves itself in an intramolecular reaction to generate the B and C forms or whether one active S1P molecule cleaves another in an intermolecular reaction. We transfected SRD-12B cells with a cDNA encoding untagged wild-type S1P or the S414A mutant. In addition, we cotransfected the wild-type or S414A version of pCMV-S1P-Myc. Membrane extracts were subjected to SDS-PAGE and immunoblotted with the antibody against the COOH terminus of S1P. Fortuitously, this antibody does not react with S1P that has a Myc tag at the COOH terminus, allowing us to visualize only the untagged S1P. As expected, when the SRD-12B cells expressed wild-type S1P, they exhibited S1P-A, -B, and -C (Fig. 8A, lane 2), whereas only S1P-A appeared when the cells expressed the S414A mutant (lane 3). When Myc-tagged wild-type S1P was co-expressed, S1P-C appeared, but S1P-B did not (lane 4). The Myc-tagged S1P by itself did not yield a visible

![Fig. 7. S1P-dependent cleavage of epitope-tagged mutant SREBP-2 in transfected SRD-12B cells. Each dish of SRD-12B cells was transfected as described under “Experimental Procedures” with 0.1 µg of pCMV-SCAP(D443N), 0.25 µg of either pTK mock vector (lanes 1, 3, 5, 7, and 9) or pTK-Myc-S1P (lanes 2, 4, 6, 8, and 10), and 3 µg of the indicated plasmid: pTK mock vector (lanes 1 and 2), pTK-HSV-BP2 (lanes 3 and 4), pTK-HSV-BP2(RS19A) (lanes 5 and 6), pTK-HSV-BP2(RSLK) (lanes 7 and 8), or pTK-HSV-BP2(RRLL) (lanes 9 and 10). Following transfection, cells were incubated in Medium B supplemented with 5% fetal calf serum. After 18 h, cells were harvested, and the nuclear extract and 105 x g membrane fractions were prepared as described under “Experimental Procedures.” Aliquots of nuclear extract (2 µg) and membrane pellets (5 µg) were subjected to SDS-PAGE on 8% gels followed by immunoblot analysis with 0.5 µg/ml anti-HSV tag antibody. The filters were exposed to film for 1 s. P and N denote the precursor and cleaved nuclear forms of SREBP-2, respectively. Molecular mass standards are expressed as kDa.](image)

![Fig. 8. S1P processes catalytically inactive S1P(S414A) in trans at Site-C, but not at Site-B, in transfected SRD-12B cells. A. Each dish of SRD-12B cells was transfected with 1 µg of either pCMV-S1P (lane 2) or pCMV-S1P(S414A) (lanes 3, 4, 6, and 7) plus 0.1 µg of either pCMV-S1P-Myc (lanes 4 and 5) or pCMV-S1P-Myc(S414A) (lanes 7 and 8) as described under “Experimental Procedures.” The total amount of DNA in all lanes was adjusted to 3 µg/dish by the addition of pcDNA3 mock vector. Following transfection, cells were incubated in Medium B supplemented with 5% fetal calf serum. After 18 h, the cells were harvested, and 105 x g membrane fractions were prepared as described under “Experimental Procedures.” Aliquots of membrane (5 µg) were subjected to SDS-PAGE on a 6% gel followed by immunoblot analysis with 2.5 µg/ml anti-Myc antibody. The filters were exposed to film for 2 s.](image)
of either pTK mock vector (lane 1) or pCMV-Myc-S1P (lanes 2 and 3) plus 3 μg of either pTK mock vector (lane 1) or pTK-HSV-BP2 (lanes 2 and 3) as described under "Experimental Procedures." After transfection, cells were refed with 5 ml of medium C (Medium B containing 5% newborn calf lipoprotein-deficient serum, 50 μg/ml sodium mevalonate) in the absence (−) or presence (+) of sterols (1 μg/ml cholesterol and 1 μg/ml of 25-hydroxycholesterol plus 10 μg/ml cholesterol added in a final concentration of 0.2% (v/v) ethanol). After 18 h, cells were harvested, and 10^5 g membrane fractions and nuclear extracts were prepared as described under "Experimental Procedures." A, aliquots of membranes (10 μg) were subjected to SDS-PAGE on a 6% gel followed by immunoblot analysis with a 1:250 dilution of anti-S1P antibody to 0.5 μg/ml of anti-HSV tag antibody. The filter was exposed to film for 45 s. B denotes the cleaved nuclear form of SREBP-2. Molecular mass markers are expressed as kDa.

The current data provide evidence that S1P, like its relatives in the subtilisin superfamily, is synthesized as an inactive precursor that must undergo autocatalytic cleavage in order to become an active protease. The membrane topology of S1P was established by a combination of protease protection assays and protein sequencing experiments. These revealed that S1P contains a 22-amino acid NH2-terminal signal sequence that directs the protein into the lumen of the ER and is cleaved, presumably by signal peptidase, to generate the form designated S1P-A. S1P-A remains attached to ER membranes by virtue of the hydrophobic membrane anchor sequence near the COOH terminus. This hydrophobic sequence is followed by a short positively charged sequence that projects into the cytosol.

The first autocatalytic cleavage of S1P occurs at Site-B, which is the Lys-Phe bond in the sequence RSLKF. This cleavage removes a 115-residue propeptide from the NH2 terminus of S1P-A. This cleavage does not occur when the active-site serine of S1P is replaced with alanine (S414A mutant), confirming that the cleavage is autocatalytic (Fig. 2). The S414A mutant is not cleaved to the B form even when cells express other copies of active S1P, indicating that Site-B must be generated by an intramolecular cleavage reaction (Fig. 8). Remarkably, the propeptide is not degraded, but rather it is secreted from the cell. Evidence that the secreted propeptide is the product of cleavage at Site-B comes from three observations: 1) mutagenesis of S1P at Site-B blocked the generation of this peptide (Fig. 5B), 2) the mobility of this peptide on SDS-PAGE is identical to that of a recombinant protein corresponding to amino acids 23–137 of S1P, and 3) the secreted propeptide is not digested by peptide N-glycosidase F, indicating that it does not contain N-linked carbohydrate (data not shown). If the propeptide were produced by cleavage at more distal sites, it should contain at least one N-linked sugar chain (see Fig. 4B).

After the propeptide is released by cleavage at Site-B, S1P-B undergoes further cleavage at Site-C, which is the Leu-Arg bond in the sequence RLLR. This cleavage also requires an active S1P enzyme because it, too, is abolished by the S414A mutation (Fig. 2). However, in contrast to Site-B cleavage, the S1P-C cleavage reaction can be catalyzed by a nearby S1P enzyme in an intermolecular reaction (Fig. 8).

The processing reaction described in this paper shows some similarities to and some differences from the processing of mammalian furin and the prohormone convertases, which have been studied extensively (for review, see Ref. 12). Like S1P, these enzymes are initially inserted into the ER lumen by means of a signal sequence, and they are anchored to the membrane by a transmembrane sequence near the COOH terminus. In the ER, these proteases process themselves intramolecularly to release propeptides of 100–150 amino acids that are similar in length, but not in sequence, to the 115-amino acid propeptide released from S1P (29). In furin, the propeptide remains associated with the enzyme, inhibiting its activity until the enzyme reaches the trans-Golgi network, where a second cleavage occurs within the propeptide, allowing it to dissociate from the enzyme, which is thereby activated (30).

This second cleavage reaction requires millimolar calcium and acidic pH, conditions that mimic those in the trans-Golgi network (30). In contrast to the findings with furin, our data indicate that the S1P propeptide is not further cleaved, but rather it is secreted intact, as judged by its migration on SDS-PAGE.

After the propeptide is cleaved, S1P-B remains endo H-sensitive, indicating that Site-B cleavage occurs in the ER. It will be important to determine whether the propeptide is released.
from S1P immediately after cleavage at Site-B or whether it remains associated, thereby inhibiting the enzyme. The conditions required to release the propeptide must also be elucidated. Our data show that the conversion of S1P-A to S1P-B and S1P-C is not regulated by sterols, but it remains possible that the release of the propeptide from S1P-B is regulated by sterols and that this regulation plays a role in modulating the activity of S1P.

The observations that S1P cleaves itself at an RSLK sequence and that it is capable of cleaving a mutant SREBP-2 with RSLK at the cleavage site broaden the previously determined RXXL consensus to RXX(L/K) (4). This is in contrast to the sequence specificity of furin and the prohormone convertases in which an arginine residue at P1 is essential (12).

Thus, it seems likely that under physiologic conditions, most of the conversion of S1P-A to S1P-B is carried out by S1P-B. We believe that the residual activity of the Site-B mutant is attributable to the S1P-C that forms under these conditions (Fig. 5). S1P-C may be able to cleave only a small amount of SREBP-2 before it leaves the ER.

SIP differs from furin and the prohormone convertases in one major way, namely, the absolute requirement in vivo for an accessory protein, SCAP (2). It will be important to determine whether SCAP directly activates SIP or whether its function is to bring SREBPs to the cellular site that houses active SIP. Recent experiments suggest that SCAP cycles between the ER and Golgi in sterol-deprived cells and that sterols cause SCAP to be retained in the ER (19). This finding supports a model in which SCAP escorts SREBPs to a cleavage site that is located in some compartment intermediate between the ER and the Golgi, where active SIP resides.

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