Secreted Site-1 Protease Cleaves Peptides Corresponding to Luminal Loop of Sterol Regulatory Element-binding Proteins*

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We describe a permanent line of Chinese hamster ovary cells transfected with a cDNA encoding a truncated form of Site-1 protease (S1P) that is secreted into the culture medium in an enzymatically active form. S1P, a subtilisin-like protease, normally cleaves the luminal loop of sterol regulatory element-binding proteins (SREBPs). This cleavage initiates the two-step proteolytic process by which the NH$_2$-terminal domains of SREBPs are released from cell membranes for translocation to the nucleus, where they activate transcription of genes involved in the biosynthesis and uptake of cholesterol and fatty acids. Truncated S1P (amino acids 1–983), produced by the transfected Chinese hamster ovary cells, lacks the COOH-terminal membrane anchor. Like native S1P, this truncated protein undergoes normal autocatalytic processing after residue 137 to release an NH$_2$-terminal propeptide, thereby generating an active enzyme, designated S1P-B. Prior to secretion, truncated S1P-B, like native S1P-B, is cleaved further after residue 186 to generate S1P-C, which is the only form that appears in the culture medium. The secreted enzyme, designated S1P(983)-C, cleaves a synthetic peptide that terminates in a 7-amino-4-methyl-coumarin fluorochrome. This peptide, RSLK-MCA, corresponds to the internal propeptide cleavage site that generates S1P-B as described in the accompanying paper (Espenshade, P. J., Cheng, D., Goldstein, J. L., and Brown, M. S. (1999), J. Biol. Chem. 274, 22795–22804). The secreted enzyme does not cleave RSVL-MCA, a peptide corresponding to the physiologic cleavage site in SREBP-2. However, S1P(983)-C does cleave after this leucine when the RSVL sequence is contained within a 16-residue peptide corresponding to the central portion of the SREBP-2 luminal loop. The catalytic activity of S1P(983)-C differs from that of furin/prohormone conver-
tases, two related proteases, in its more alkaline pH optimum (pH 7–8), its relative resistance to calcium chal-
oling agents, and its ability to cleave after lysine or leucine rather than arginine. These data provide direct biochemical evidence that S1P is the protease that cleaves SREBPs and thereby functions to control lipid biosynthesis and uptake in animal cells.

Site-1 protease (S1P) is a mammalian subtilisin-related ser-
ine protease that cleaves sterol regulatory element-binding pro-
teins (SREBPs) and initiates the release of their NH$_2$-
terminal domains from membranes (1). The NH$_2$-
terminal domains enter the nucleus, where they enhance transcription of multiple genes encoding enzymes of cholesterol and fatty acid biosynthesis and the low density lipoprotein receptor. Excess cholesterol inhibits its own synthesis by blocking the activity of S1P, thereby preventing the NH$_2$-
terminal domains of SREBPs from entering the nucleus. This feedback inhibition controls the cholesterol content of cells and plasma (2).

The RSVLS target sequence for S1P is located in a luminal loop between two membrane-spanning helices of SREBP-2 (3). The NH$_2$-terminal and COOH-terminal domains of SREBPs extend into the cytosol, and the luminal loop projects into the endoplasmic reticulum (ER) and nuclear envelope. Cleavage by S1P separates SREBP-2 into two halves. This allows a second protease to cleave at Site-2, which is within the first transmembrane helix, thereby liberating the NH$_2$-terminal domain from the membrane (4, 5). SREBPs are complexed with a polytopic membrane protein called SREBP cleavage-activating protein (SCAP), the presence of which is required for S1P to act within cells (6, 7). Sterols inhibit Site-1 cleavage by abolishing the activity of SCAP.

The S1P cleavage reaction has been studied in intact cells that were transfected with cDNAs encoding wild-type or mu-
tant forms of SREBP-2. S1P cleaves SREBP-2 between the leucine and serine of the sequence RSVLS (8). Cleavage absolutely requires a basic residue at the P4 position. Either of the serines or the valine can be replaced with alanine without affecting cleavage, but a leucine to valine replacement abolishes cleavage.

In an accompanying paper (9), we show that the S1P precursor is inserted into the ER lumen by virtue of a cleaved NH$_2$-
terminal signal sequence. The protein remains attached to ER membranes through a hydrophobic transmembrane segment near the COOH terminus. This segment is followed by a short positively charged sequence that extends into the cytosol. Signal peptidase generates the pro form of S1P, which is designated S1P-A. This form is inactive. S1P-A is activated by intramolecular cleavage at Site-B, which follows lysine-137. The hamster sequence at this site is RSLKF. This releases a propeptide of 115 amino acids and activates the enzyme. This

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1 The abbreviations used are: S1P, Site-1 protease; S2P, Site-2 protease; AMC, 7-amino-4-methyl-coumarin; CHO, Chinese hamster ovary; CMV, cytomegalovirus; endo H, endoglycosidase H; MCA, 4-methylcoumaryl-7-amide; PAGE, polyacrylamide gel electrophoresis; SREBP, sterol regulatory element-binding protein; SCAP, SREBP cleavage-activating protein; ER, endoplasmic reticulum; HPLC, high performance liquid chromatography; kb, kilobase; Mes, 4-morpholinoneethanesulfonic acid.
SREBP Site-1 Protease: In Vitro Assay

propeptide is secreted from cells, apparently in an intact form. Subsequently, S1P undergoes cleavage after the second leucine of the sequence RRLRR, which removes an additional 49 amino acids and generates S1P-C (amino acids 187–1052). The carbohydrates of S1P-A and S1P-B remain endo H-sensitive, indicating that these proteins are located in pre-Golgi compartments. S1P-C is endo H-resistant, suggesting that it resides in the Golgi or more distal compartments. Mutation of the active site serine of S1P (S414A) inactivates the enzyme and prevents generation of either S1P-B or S1P-C (9), indicating that these cleavages are autocatalytic.

Studies of S1P are facilitated by the availability of SRD-12B cells, a line of mutant Chinese hamster ovary (CHO) cells that fails to produce S1P mRNA and protein, owing to mutations in both copies of the S1P gene (1, 10). As a result of this defect, SRD-12B cells are unable to release SREBPs from membranes, and they fail to transcribe multiple genes encoding the low density lipoprotein receptor and the enzymes of cholesterol and unsaturated fatty acid biosynthesis. These cells can grow only if they are supplied with exogenous sources of cholesterol and oleate.

The activity of S1P in cleaving SREBP has been studied so far only in intact cells. To understand this reaction more completely and to show directly that S1P is able to cleave the SREBP-2 recognition sequence, in the current studies, we prepared a permanent line of SRD-12B cells that expresses a truncated form of S1P that lacks the COOH-terminal membrane anchor. Despite the lack of membrane attachment, this truncated form of S1P processes SREBPs normally and alleviates the lipid growth requirements of the cells. Truncated S1P is secreted from the cells and can be isolated from the culture medium. This secreted enzyme cleaves peptide substrates corresponding to the recognition site in SREBP-2 and to the internal recognition site within S1P that releases the propeptide. These developments have permitted us to characterize the catalytic activity of S1P in vitro.

EXPERIMENTAL PROCEDURES

Materials—We obtained serum-free CHO-S-SFM II medium from Life Technologies, Inc.; Pefabloc SC, phenylmethylsulfonyl fluoride, (4-aminophenyl)-methanesulfonyl fluoride, leupeptin, and pepstatin from Roche Molecular Biochemicals; N-acetyl-leucinal-leucinal-norleucinal from Calbiochem; and aprotinin from Sigma. Ac-Ser-Gly-Arg-Ser-Val-Leu-MCA, and Peninsula Laboratories, Inc. (Belmont, CA), respectively. Peptides Ac-Val-Phe-Arg-Ser-Leu-Lys-MCA, Ac-Val-Phe-Ala-Ser-Leu-Lys-MCA, Ac-Val-Phe-Ser-Arg-Ser-Val-Leu-Lys-MCA, Ac-Arg-Ser-Ser-Leu-Lys-MCA, and Ac-Arg-Ser-Val-Leu-MCA were synthesized by Tularik Inc. (South San Francisco, CA) by solution coupling of the appropriate fully deprotected peptide fragment with Lys-MCA, Arg-MCA, or Leu-MCA followed by reverse-phase HPLC purification. Peptides NH2-Arg-Lys-Val-Phe-Arg-Ser-Leu-Lys-Phe-Ala-Glu-Ser-Asp-Pro-Ile-Val-COOH and NH2-His-Ser-Gly-Ser-Gly-Ser-Arg-Val-Leu-Ser-Asp-Ser-Glu-Ser-Gly-Arg-COOH were synthesized in Dallas using standard Fmoc (N-(9-fluorenylmethoxycarbonyl)) solid phase synthesis chemistry on a Rainin Symphony multiplex peptide synthesiser (Washburn, MA). IgG-TD4, a mouse monoclonal antibody directed against the NH2-terminal domain of hamster SREBP-2 (2-mono acids 32–250), was prepared as described previously (11). Other materials were obtained from sources described in the accompanying article (9).

Construction of Plasmids—pcMV-SIP(1052)-Myc-His encodes an 1102-amino acid fusion protein that contains full-length S1P. It consists of an initiator methionine, amino acids 2–1052 of hamster S1P (1), three novel amino acids (GGR) encoded by the sequence of the NotI restriction enzyme digestion on the human c-Myc protein (EQKILISEELDGQGEKILISEDGDPRFEQKILISEDLD), five novel amino acids (DMHTG) encoded by linker sequences, and six consecutive histidines. Expression is driven by the CMV promoter/enhancer. pcMV-SIP(1052)-Myc-His was constructed in three steps, as follows. First, an intermediate plasmid (plasmid 1) was constructed by ligation of three DNA fragments: 1) a ~5.4-kb fragment released from the BamHI and NotI digestion of pcDNA3 (Invitrogen), 2) a ~4-kb fragment released from the EcoRI and NotI digestion of pcMV-SIP (1), and 3) a ~150-base pair fragment released from the BamHI and EcoRI digestion of a polynucleotide chain reaction-amplified product obtained from the pCMV-SIP using the following primers: 5’ primer, 5'-CGGGAGTCATGGATCCTCATTCAACAATCTTGCC-3’ and 3’ primer, 5'-GGAGAATTCACCTCTTCAAGA-TGAG-3’. Second, an intermediate plasmid (plasmid 2) was constructed by ligation of the following three fragments: a ~5.5-kb fragment released from a BamHI and NotI digestion of pcDNA31/Myc-His (+) B (Invitrogen); a ~2.6-kb fragment released from a BamHI and NotI digestion of plasmid 1; and 3) a ~550-base pair fragment released from the NdeI and NotI digestion of a polynucleotide chain reaction-amplified product obtained from the pCMV-SIP template using the following primers: 5’ primer, 5'-TTTCACTACAATCATGATGGGTAAGACCTTCATG-3’ and 3’ primer, 5'-TAGACGTCAGGGCGCG-CACCACACGGGTCGCTCACCACCCAGATCCTCTTCTGAGATGAGT-3’ were annealed. These oligonucleotides correspond to two additional copies of the c-Myc 9E10 epitope tag. The annealed oligonucleotides were cloned into the ~5-kb fragment released from the ApaI and NotI digestion of intermediate plasmid 2. The plasmid resulting from this ligation is designated pCMV-SIP(102)-Myc-His.

pCMV-SIP(983)-Myc-His encodes the same fusion protein as does pCMV-SIP(1052)-Myc-His, except that the DNA encoding the last 69 amino acids of S1P (residues 984–1052) was deleted so as to remove the membrane anchor and COOH-terminal tail. The strategy for the construction of this plasmid was similar to that for pCMV-SIP(1052)-Myc-His except for the second step, in which a ~300-base pair fragment was amplified from pcMV-SIP using the following primers: 3’-primer, 5’-TACCTGGGCGCGCGCCTTCTGGGTTGAGGCGCAGCATGATC-3’ and 5’-primer, same as described above.

All of the polynucleotide chain reaction-amplified fragments and ligation junctions in each step of the above constructions were confirmed by DNA sequencing.

Stable Transfection of SRD-12B Cells with Epitope-tagged S1P—On day 0, cholesterol auxotrophic SRD-12B cells were plated at a density of 5 × 10⁴ cells/100-mm dish in Medium B (9) supplemented with 5% fetal calf serum, 5 μg/ml cholesterol, 1 mM sodium mevalonate, and 20 μg/ml sodium oleate. On day 1, cells were transfected with 5 μg of either pCMV-SIP(1052)-Myc-His or pCMV-SIP(983)-Myc-His using an MBS kit (Stratagene) and cultured overnight in Medium B supplemented with 5% fetal calf serum. On day 2, the medium was switched to Medium B supplemented with 5% fetal calf lipoprotein-deficient serum without cholesterol. The medium was changed every second day until individual colonies were visible on day 11. Stable expression of S1P-Myc permitted the growth of SRD-12B cells in the absence of sterols (1). Single cell clones that stably expressed S1P were isolated by limiting dilution cloning, analyzed for S1P expression by immunoblotting with the anti-Myc (9E10 clone) monoclonal antibody. The resulting cell lines expressing S1P(1052)-Myc-His (TR-3109 cells) and S1P(983)-Myc-His (TR-3117 cells) are designated S1P(1052) and S1P(983) cells, respectively.

Purification of S1P(983)-C—Stock cultures of S1P(983) cells were grown in 800-cm² roller bottles in Medium B (9) supplemented with 5% (v/v) newborn calf lipoprotein-deficient serum and 500 μg/ml G418. On day 0, 10 roller bottles of S1P(983) cells were seeded at a density of ~10⁴ cells/roller bottle. On day 2, the medium was replaced with 100 ml of serum-free medium CHO-S-SFM II. The medium from each roller bottle was collected daily from day 3 to day 7. The collected medium was pooled, filtered through 0.45-μm cellulose acetate acid protein binding membrane filter units (Corning Costar), and stored at 4 °C for 0–4 days. On day 7, the filtered medium was adjusted to pH 8.0 by addition of Tris-HCl at a final concentration of 25 mM and then loaded onto four parallel 5-mL Ni-NTA agarose columns (Qiagen, Inc.). The concentrated solution was supplemented with an equal volume of 100% glycerol and stored in multiple aliquots at −20 °C without loss of activity for at least 4 months. Ten roller bottles of cells
yielded 1 mg of protein.

Fluorogenic Peptide Assay for S1P Activity—S1P activity was measured fluorometrically with MCA-conjugated peptidyl substrates. Each reaction was carried out in 0.2 ml of assay buffer (25 mM Tris, 25 mM Mes, 25 mM acetic acid, and 1 mM CaCl2 adjusted to pH 8.0 with concentrated NaOH). Reactions contained MCA-peptide (final concentration 100 μM, added in 2 μl DMSO) and 1.5–5 μg of purified S1P(983)-C. After incubation for 0.5–5 h at 37 °C, each reaction was terminated by addition of 1 ml of ice-cold 5 mM sodium EDTA. The liberated 7-amino-4-methyl-coumarin (AMC) was measured with a Perkin-Elmer LS-30 luminescence spectrometer (360 nm excitation, 460 nm emission). A standard curve of fluorescence intensity was generated with different concentrations of AMC (Promega).

RESULTS

Fig. 1A diagrams the structure of the S1P constructs that were used in these studies. The S1P signal sequence ends at residue 22. The propeptide cleavage site (Site-B) is after residue 137. Cleavage at this site generates S1P-B. This is followed by a second cleavage after residue 186 to generate S1P-C. The active site serine is at residue 414, and the transmembrane anchor sequence begins at residue 997. The wild-type protein terminates at residue 1052. We also prepared a cDNA encoding a truncated form of S1P that terminates after residue 983,
deleting the transmembrane sequence and the COOH-terminal tail. Three copies of a Myc epitope tag and six consecutive histidines are added at the COOH terminus of both proteins.

cDNAs encoding the proteins illustrated in Fig. 1A were introduced into SRD-12B cells, which lack S1P (10). Permanent cell lines were grown in the absence of cholesterol and oleate, a protocol that selects for cells that express functional S1P. The resulting permanent lines that express full-length and truncated protein are called S1P(1052) and S1P(983), respectively.

To quantify expression of the transfected S1P, the cells were homogenized, and a crude membrane pellet was subjected to SDS-PAGE and immunoblotted with an antibody against the Myc tag (Fig. 1B). In order to study the regulation of S1P activity, we incubated the cells in the absence of sterols with or without the 3-hydroxy-3-methylglutaryl CoA reductase inhibitor compactin. S1P is expected to be active under both conditions. We incubated parallel dishes in the presence of compactin plus or minus sterols. Without the 3-hydroxy-3-methylglutaryl CoA reductase inhibitor compactin, S1P is expected to be active under both conditions.

As shown in Fig. 1C, nuclear extracts from CHO-7 cells contained detectable amounts of the NH2-terminal segment of SREBP-2, as determined by immunoblotting when the cells were incubated in the absence of sterols (lane 1). The amount was increased when the cells were further deprived of sterols by treatment with compactin (lane 2). The band disappeared when the compactin-treated cells were incubated with sterols (lane 2). The SRD-12B cells failed to exhibit nuclear SREBP-2 under any of these conditions (lanes 4–6). In the absence of sterols, nuclear SREBP-2 was found in both the S1P(1052) cells and the S1P(983) cells in either the absence or presence of compactin (lanes 7, 8, 10, 11). In both cases, nuclear SREBP-2 was abolished by addition of sterols (lanes 9 and 12). These data indicate that the truncated S1P and the full-length S1P are active and regulated normally by sterols in the transfected cells. The bottom panel of Fig. 1C confirms that membranes from all of the cells contained the precursor form of SREBP-2.

Fig. 2 shows an experiment designed to determine whether the transfected cells secreted S1P and to assess the form of the secreted protein. For this purpose, the cells were incubated in fresh medium for 24 h, after which the medium was removed and the cells were lysed in a detergent buffer. Aliquots of the cell lysate corresponding to 0.1 dish of cells and aliquots of concentrated medium also corresponding to 0.1 dish of cells were subjected to SDS-PAGE and blotted with anti-Myc (Fig. 2A). In the S1P(1052) cells, all immunoreactive protein was found in the lysate (Fig. 2A, lane 3), and none was in the medium (lane 4). The lysate contained three bands corresponding to S1P-A, -B, and -C. In the S1P(983) cells, the lysate contained two bands corresponding to the sizes expected for S1P(983)-A and S1P(983)-B (Fig. 2A, lane 5). The medium contained abundant amounts of a protein corresponding to the expected size of S1P(983)-C (Fig. 2A, lane 6). At the 24 h time point, the total amount of S1P(983)-C in the medium was roughly equal to the amount of S1P(983)-A plus S1P(983)-B in the lysate.

Consistent with the findings in the accompanying paper (9), in the S1P(1052) cells, S1P-A, -B, and -C all showed increased mobility after treatment with peptide N-glycosidase F (Fig. 2B, lane 2), indicating that all three forms contain N-linked carbohydrates. Endo H increased the mobility only of S1P-A and S1P-B. Endo H-treated S1P-B overlapped with S1P-C, which was unaffected by endo H (lane 3). In lysates from the S1P(983) cells, the A and B forms were both sensitive to endo H as well as to peptide N-glycosidase F. No C form was visible (Fig. 2B, lanes 6 and 7). The medium from these cells contained a protein that corresponded in size to S1P(983)-C (lane 9). This protein was sensitive to peptide N-glycosidase F (lane 10), but resistant to endo H (lane 11). To confirm the identity of this band, we grew the S1P(983) cells in bulk in roller bottles and purified the protein from the medium by nickel affinity chromatography, taking advantage of the His6 tag. Fig. 2C shows a Coomassie Blue stain of an SDS-polyacrylamide gel containing this purified protein. A corresponding band was excised from an unstained parallel gel and subjected to Edman degradation, revealing the NH2-terminal sequence shown in Fig. 2C. This sequence corresponds to the product expected from cleavage at Site-C (see Fig. 4 of Ref. 9).

To characterize the catalytic activity of the secreted enzyme, we prepared a series of peptides that contain a COOH-terminal amide of AMC at the COOH terminus. AMC does not show fluorescence at 460 nm unless it has been released from the peptide through proteolysis. Similar substrates have been used to assay furin, prohormone convertases, and other subtilisin-related enzymes (12). Fig. 3A shows the fluorescence observed when the peptide Ac-VRFRSL-MCA was incubated with purified secreted S1P(983)-C. The sequence of this peptide corresponds to the sequence at Site-B of S1P, which is known to be cleaved by S1P in an autocatalytic reaction (9). Fluorescence rose linearly with time for 5 h. Fig. 3B shows a saturation curve with the same substrate performed at the 4-h time point. The inset shows a Lineweaver-Burk plot of these data, which yielded a K_m of 100 μM and a V_max of 1.6 nmol/min/mg of protein. The K_m was only 0.16 mol/min/mol of enzyme, which is extremely low, but is sufficient for preliminary characterization. Cleavage was absolutely dependent on the arginine at the P4 position because there was no detectable cleavage of the peptide Ac-VFASL-MCA (Fig. 3B). Surprisingly, the enzyme did not cleave the MCA-peptide corresponding to Site-1 in SREBP-2 (Ac-SGRSVL-MCA). The maximal rate of cleavage of Ac-VRFRSL-MCA was observed at pH 8, and there was very little cleavage at pH 6 (Fig. 3C). This differs from the observations with furin and the prohormone convertases, the pH optima of which are generally in the range of 6–7 (12).

To further explore the recognition requirements for the peptide-MCA assay, we prepared a series of MCA-coupled peptides of varying sequence and incubated them with purified S1P(983)-C. Shortening the Ac-VRFRSL-MCA peptide by two residues reduced its susceptibility to cleavage by about 50% (Table I). Ac-BSVL-MCA peptides ranging in length from four to eight residues were not cleaved. Importantly, S1P did not cleave MCA peptides that corresponded to the recognition sequence for the prohormone convertases, i.e. Ac-VFRSSRR-MCA or Boc-RVRR-MCA. The latter peptide has been used for in vitro assays of furin (13).
ylmethylsulfonyl fluoride. The enzyme was also resistant to leupeptin, pepstatin, aprotinin, and \(N\)-acetyl-leucinal-leucinal-norleucinal. It was partially inhibited by EDTA, EGTA, and 1,10-phenanthroline, but only at extremely high concentrations. The same was true of dithiothreitol. In experiments not shown, we found that the enzyme reaction was not affected by omission of calcium during the purification procedure and in the assay buffer. Moreover, we observed that the reaction was inhibited by calcium concentrations above 3 mM.

The low activity of the purified S1P raised the possibility that the true activity might be attributable to a trace amount of a more active contaminating enzyme that could not be visualized on the Coomassie-stained gels. To rule out this possibility, we performed an immunoprecipitation experiment (Fig. 4). Purified S1P(983)-C was incubated with agarose beads containing a control antibody or anti-Myc. After centrifugation, the supernatant and pellet fractions were subjected to SDS-PAGE and blotted with anti-Myc. As shown in Fig. 4A, anti-Myc, but not

**Fig. 2.** Characterization of S1P(983)-C secreted by S1P(983) cells. A, immunoblot analysis of total cell lysate and medium of SRD-12B, S1P(1052), and S1P(983) cells. On day 0, cells were set up for experiments at \(7 \times 10^5\) cells/60-mm dish as described in Fig. 1. On day 1, the medium was changed to Medium B supplemented with 1% newborn calf lipoprotein-deficient serum. On day 2, the cells were washed and lysed with Buffer A as described in the accompanying paper (9). The medium was treated with cold 80% acetone, and the pellet was resuspended in 1× SDS gel loading buffer (9). Aliquots of the cell lysate (15 μg of protein from 0.1 dish of cells) and the acetone-precipitated proteins from the medium were subjected to SDS-PAGE and immunoblotted with 0.5 mg/ml monoclonal 9E10 anti-Myc antibody. The filter was exposed to film for 1s. L and M denote cell lysate and medium fractions, respectively. A, B, and C denote three forms of S1P. B, glycosidase treatment of cellular and secreted S1P. Aliquots (10 μl) of total cell lysate and medium fraction (prepared as in A) were boiled as described in the accompanying paper (9) and treated with either 0.04 IU of peptide-N-glycosidase F (PNGase F) or 0.25 IU of endo H as indicated. The samples were then subjected to SDS-PAGE and immunoblot analysis as described in A. C, Coomassie Blue stain of purified S1P(983)-C. On day 0, 10 roller bottles of S1P(983) cells were set up as described under “Experimental Procedures.” On day 2, the medium was switched to serum-free CHO-S-SFM II medium. The pooled medium collected from day 3 to day 7 was loaded onto Ni-NTA agarose columns, and chromatography was performed as described under “Experimental Procedures.” The eluted protein was concentrated to 2 ml, and 10 μl- aliquots were subjected to SDS-PAGE and Coomassie Blue staining. The band in a parallel gel corresponding to the Coomassie Blue-stained protein was transferred to a polyvinylidene fluoride membrane, cut from the membrane, and subjected to NH\(_2\)-terminal sequencing by Edman degradation as described in the accompanying paper (9). Molecular mass standards are expressed in kDa.
the control antibody, precipitated the S1P(983)-C. The supernatant and pellet fractions were also assayed for the ability to cleave Ac-VFRSLK-MCA (Fig. 4B). When the material was treated with a control antibody, the enzyme activity was in the supernatant. When anti-Myc was used, the activity was all found in the pellet. These data demonstrate that the cleavage of the peptide substrate in this assay is due to the activity of S1P(983)-C.

The failure of S1P(983)-C to cleave the RSVL-MCA peptides might have been due to an inability of the enzyme to recognize the RSVL sequence, or alternatively it might be due to the resistance of the peptide-MCA bond to cleavage. To distinguish between these possibilities, we prepared 16-residue peptides composed of the amino acids surrounding the internal cleavage Site-B in S1P (i.e. RSLK) or Site-1 in SREBP-2 (i.e. RSVL). The peptides were incubated with or without purified S1P(983)-C, and the products were separated by HPLC. In the absence of S1P, both peptides gave single predominant peaks on the HPLC (Fig. 5, A and C). After incubation with S1P(983)-C, these peptides were each cleaved to generate two smaller peptides (Fig. 5, B and D). These peptides were examined by mass spectrometry and the sequences were found to correspond to those predicted from cleavage after RSLK and RSVL, respectively. Thus, S1P(983)-C has the potential to cleave Site-1 in SREBP-2.

**DISCUSSION**

The current studies demonstrate that a secreted, truncated form of S1P is able to cleave peptide substrates corresponding to Site-1 in SREBP-2 and to the internal propeptide cleavage site within S1P itself. The characteristics of these in vitro reactions differed importantly from the activities previously observed for furin and the prohormone convertases, which are the only subtilisin-like enzymes previously characterized in animal cells.

The studies of secreted S1P were facilitated by the use of SRD-12B cells, the auxotrophic growth requirements of which

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**TABLE I**

Comparative cleavage of fluorogenic synthetic peptidyl substrates by purified S1P(983)-C

| Substrate     | Relative activity |
|---------------|------------------|
| Ac-VFRSLK-MCA | 1.0              |
| Ac-RSLK-MCA   | 0.47             |
| Ac-VFSKL-MCA  | <0.1             |
| Ac-SGGRSVL-MCA| <0.1             |
| Ac-RSVL-MCA   | <0.1             |
| Ac-VFRR-MCA   | <0.1             |
| Boc-RVRR-MCA  | <0.1             |

The indicated fluorogenic peptide-MCA (100 μM) was incubated with 3 μg of purified S1P(983)-C at 37 °C for 5 h as described under “Experimental Procedures.” The S1P activity of Ac-VFRSLK-MCA (930 pmol/min/mg of protein) was set at 1.0, and the activity of the other substrates was expressed relative to this value. Each value represents the average of duplicate incubations, which varied from each other by <10%.

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**TABLE II**

S1P(983)-C mediated cleavage of Ac-VFRSLK-MCA: effect of protease inhibitors

| Addition       | Concentration | AMC liberated |
|----------------|---------------|---------------|
|                | μM            | pmol/tube     | % control |
| None           |               | 839           | 100       |
| Pefabloc®      | 1             | 731           | 87        |
|                | 3             | 366           | 44        |
|                | 10            | 60            | 7         |
| PMSF           | 1             | 853           | 102       |
|                | 10            | 690           | 82        |
| APMSF          | 1             | 793           | 95        |
| PMSF/APMSF     | 10/1          | 745           | 89        |
| Leupeptin      | 1             | 754           | 90        |
| Pepstatin      | 0.01          | 798           | 95        |
| Aprotinin      | 0.0003        | 745           | 89        |
| ALLN           | 0.1           | 811           | 97        |
| EDTA           | 3             | 802           | 96        |
|                | 30            | 303           | 36        |
| EGTA           | 3             | 609           | 73        |
|                | 30            | 305           | 36        |
| 1,10-Phenanthroline | 3 | 361     | 43        |
|                | 10            | 253           | 30        |
| EDTA/EGTA/1,10-phenanthroline | 3/3/3 | 163 | 19        |
| Dithiothreitol | 0.1           | 615           | 73        |
|                | 1             | 323           | 38        |
|                | 10            | 99            | 12        |

**FIG. 4.** Immunoprecipitation of S1P(983)-C cleavage activity. A, immunoblot analysis of S1P(983)-C after immunoprecipitation. Aliquots (200 μg of protein) of monoclonal 9E10 anti-Myc antibody or an irrelevant IgG-2001 (15) were each incubated with 100 μl of protein A/G plus-agarose beads (Santa Cruz Biotechnology, Inc.) for 2 h at 4 °C. The beads were pelleted by centrifugation at 3000 × g for 10 min at 4 °C and then washed four times with 1 ml of assay buffer (25 mM Tris, 25 mM Mes, 25 mM acetic acid, 1 mM CaCl₂, at pH 8). The washed beads were incubated with 15 μg of Ni-NTA agarose column-purified Myc-tagged S1P(983)-C in 1 ml of assay buffer for 2 h at 4 °C, after which the beads were pelleted at 3000 × g for 10 min, washed four times with 1 ml of assay buffer and resuspended in 1 ml of assay buffer. Aliquots (10 μl) of the supernatant (S), pellet (P), and input material were subjected to SDS-PAGE and immunoblot analysis with 0.5 μg/ml of the anti-Myc antibody. The filter was exposed to film for 1 s. B, fluorogenic peptide assay of S1P activity after immunoprecipitation. Aliquots (0.2 ml) of the supernatant, the resuspended agarose beads, and input material were incubated with 100 μM Ac-VFRSLK-MCA for 5 h at 37 °C under standard assay conditions. The data are expressed as the amount of AMC liberated by the input material, which was set at 100%. Each value is the average of duplicate assays.
could be overcome by expression of S1P, even when the protein was truncated so as to delete the membrane-spanning and cytosolic domains. After transfecting SRD-12B cells with expression vectors encoding truncated S1P, we were able to select for high level expression by growing the cells in the absence of cholesterol, oleate, and mevalonate. After incubation of the cells for 5 days in lipoprotein-deficient medium, the concentration of truncated S1P reached approximately 0.2 mg/liter in the extracellular fluid. The protein contained a hexahistidine tag that allowed purification by nickel affinity chromatography.

Following cleavage by signal peptidase, the S1P proenzyme, designated S1P-A, undergoes two further cleavages to generate S1P-B and S1P-C (9). Experiments with mutant forms of S1P suggest that S1P-A is inactive and that S1P-B is responsible for most of the physiologically relevant cleavage of SREBPs. S1P-C, although catalytically active, may not cleave SREBPs within cells. This conclusion follows from experiments performed with cells transfected with cDNAs encoding altered forms of SREBP-2 that contain N-linked glycosylation sites in the luminal loop (8). These studies show that SREBP-2 is cleaved when the N-linked sugars are still in the endo H-sensitive form, i.e., before the protein has reached the Golgi apparatus. The carbohydrates on S1P-A and S1P-B are also in the endo H-sensitive form, indicating that these enzymes are potentially in the same compartment as the substrate SREBPs. In contrast, the carbohydrates on S1P-C are in the endo H-resistant form, indicating that S1P-C is either formed in the Golgi, or else it is transported to the Golgi immediately after it is formed. In either event, most of the S1P-C is not in the compartment where SREBP cleavage takes place.

In the current studies, we found that S1P(983)-C was the only form of truncated S1P that was secreted from transfected cells. Truncated S1P(983)-A and S1P(983)-B were found only within the cells (Fig. 2). These proteins were bound to membranes, even though they lacked membrane anchors. These data suggest that S1P(983)-A and S1P(983)-B contain a sequence that allows them to bind to a membrane protein that retains them in a pre-Golgi compartment. S1P may move to the Golgi only after cleavage at Site-C. A precedent for this finding is observed in the case of furin, in which the precursor is retained in a pre-Golgi compartment by a saturable mechanism, which has been suggested to consist of binding to the ER protein calnexin (reviewed in Ref. 12). A similar mechanism may be responsible for the retention of S1P-A and S1P-B because immunoprecipitation studies demonstrated that calnexin was brought down from cell extracts by an antibody against epitope-tagged S1P (9).

Even after the propeptide cleavage reaction has taken place, furin remains inactive because the propeptide remains attached to the enzyme in a noncovalent manner. Furin becomes active only after it reaches an acidic compartment where a further cleavage occurs within the propeptide, allowing its release from the enzyme (12). We do not yet know whether the propeptide dissociates from S1P immediately after cleavage at Site-B or whether it remains attached until some subsequent event occurs.

Although secreted S1P(983)-C had definite catalytic activity toward peptide-MCA conjugates, the rate of cleavage was very slow. The maximal $K_{cat}$ was only 0.16 mol/min/mol of enzyme. This is about $\frac{1}{10}$ the value observed by others for furin in a similar assay (1.6 mol/min/mol of enzyme (13)).

One reason for this low activity may be the absence of SCAP in the in vitro assay. SCAP binds to SREBPs and is required for Site-1 cleavage in intact cells (7). S1P differs from furin and the other prohormone convertases in its absolute requirement for SCAP in vivo (1, 7). We do not know whether SCAP directly activates S1P or whether its only function is to transport SREBPs to the cellular compartment that contains S1P. If SCAP does activate the enzyme, then we may not be able to achieve full enzymatic activity in vitro until we find some way to add purified SCAP into the assay. SCAP interacts with SREBPs primarily on the cytoplasmic side of the membrane through the binding of the WD-repeat segment of SCAP to the COOH-terminal regulatory segment of SREBPs (6). In order to achieve this interaction in vitro, it may be necessary to use full-length SREBP as substrate.

The in vitro peptide cleavage assays revealed several additional aspects in which S1P differs from furin and the prohormone convertases. In addition to the difference in pH optimum that was mentioned under “Results,” furin has an absolute requirement for calcium, and it is totally inhibited by EDTA (12, 13). S1P did not require calcium in the assay medium, and it was only weakly inhibited by high concentrations of calcium chelators. This difference may relate to the difference in the compartments in which the two enzymes act. Furin acts in the distal Golgi apparatus and in secretory vesicles where the pH is acidic and the calcium concentration is high. S1P is believed to act in a pre-Golgi compartment that is likely to have a neutral pH and a low calcium concentration.

Purified S1P is also distinctive in its ability to cleave MCA peptides with lysine at the P1 position. Furin and all the known prohormone convertases have an absolute requirement for arginine at the P1 position, and lysine cannot substitute for this arginine (12). In contrast, S1P cleaves itself after the lysine of prohormone convertases. In addition to the difference in pH optimum, there are also other differences in which S1P differs from furin and the other prohormone convertases. For example, S1P differs in its requirement for calcium, and it is totally inhibited by EDTA (12, 13). S1P did not require calcium in the assay medium, and it was only weakly inhibited by high concentrations of calcium chelators. This difference may relate to the difference in the compartments in which the two enzymes act. Furin acts in the distal Golgi apparatus and in secretory vesicles where the pH is acidic and the calcium concentration is high. S1P is believed to act in a pre-Golgi compartment that is likely to have a neutral pH and a low calcium concentration.

Purified S1P did not cleave the RSVL-MCA peptide, which corresponds to Site-1 in SREBP-2, but it did cleave this sequence when it was located at the center of a 16-residue peptide representing the SREBP-2 sequence. It is possible that the presence of the MCA group reduces the affinity of S1P for MCA-conjugated peptides when leucine, in contrast to lysine, is at the P1 position. These observations are consistent with the
recent report by Seidah et al. (14), who described the cloning of a human subtilisin-related cDNA, called SKI-1, that is identical to S1P. These authors stated that they had obtained preliminary data showing that culture medium from cells infected with a recombinant vaccinia virus encoding full-length SKI-1 was unable to cleave the RSVL-MCA peptide but was able to cleave between the RSVL↓S sequence when it was contained in a 27-amino acid synthetic peptide corresponding to the luminal loop sequence of human SREBP-2.

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