Cleavage Site for Sterol-regulated Protease Localized to a Leu-Ser Bond in the Lumenal Loop of Sterol Regulatory Element-binding Protein-2*

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A sterol-regulated protease initiates release of the NH2-terminal segments of sterol regulatory element-binding proteins (SREBPs) from cell membranes, thereby allowing them to enter the nucleus and to stimulate transcription of genes involved in the uptake and synthesis of cholesterol and fatty acids. Using SREBP-2 as a prototype, we here identify the site of sterol-regulated cleavage as the Leu522-Ser523 bond in the middle of the 31-residue hydrophilic loop that projects into the lumen of the endoplasmic reticulum and nuclear envelope. This site was identified through use of a vector encoding an SREBP-2/Ras fusion protein with a triple epitope tag that allowed immunoprecipitation of the cleaved COOH-terminal fragment. The NH2 terminus of this fragment was pinpointed by radiochemical sequencing after replacement of selected codons with methionine. Alanine scanning mutagenesis revealed that only two amino acids are necessary for recognition by the sterol-regulated protease: 1) the leucine at the cleavage site (leucine 522), and 2) the arginine at the P4 position (arginine 519). These define a tetrapeptide sequence, RXXL, that is necessary for cleavage. Cleavage was not affected when the second transmembrane helix of SREBP-2 was replaced with the membrane-spanning region of the low density lipoprotein receptor, indicating that this sequence is not required for regulation. Glycosylation-site insertion experiments confirmed that leucine 522 is located in the lumen of the endoplasmic reticulum. We conclude that the sterol-regulated protease is a novel enzyme whose active site faces the lumen of the nuclear envelope, endoplasmic reticulum, or another membrane organelle to which the SREBPs may be transported before cleavage.

Proteolytic processing of sterol regulatory element-binding proteins (SREBPs)† controls the metabolism of cholesterol and fatty acids in animal cells (1–3). SREBPs are transcription factors that are bound to membranes of the ER and nuclear envelope. Each SREBP is composed of three segments: 1) an NH2-terminal segment of ~485 amino acids that is a transcription factor of the basic helix-loop-helix-leucine zipper family, 2) a membrane attachment segment of ~75 amino acids composed of two membrane-spanning sequences separated by a short hydrophilic loop of 31 amino acids, and 3) a COOH-terminal segment of ~585 amino acids that plays a regulatory role. The proteins are oriented so that the NH2- and COOH-terminal segments project into the cytoplasm, and only the short hydrophilic loop projects into the lumen of the ER or nuclear envelope (4).

Before it can activate transcription, the NH2-terminal segment is released from the membrane in a complex two-step proteolytic sequence (2, 3). First, a protease cleaves the protein at Site-1, which is near an arginine in the luminal loop, thereby breaking the attachment between the two transmembrane sequences. This allows a second protease to cleave the protein at Site-2, which is near the middle of the first transmembrane sequence (2, 3). The NH2-terminal fragment leaves the membrane with a portion of the first transmembrane sequence still attached. It enters the nucleus, where it activates transcription of genes encoding the LDL receptor (5, 6), several enzymes of cholesterol biosynthesis (3-hydroxy-3-methylglutaryl coenzyme A synthase (5, 6), 3-hydroxy-3-methylglutaryl coenzyme A reductase (7), farnesyl diprophosphate synthase (8), and squalene synthase (9)), and enzymes of fatty acid biosynthesis (10, 11) and desaturation (12). The net result is to increase the cell’s supply of cholesterol and fatty acids.

The Site-1 protease is the target of feedback regulation by cholesterol and other sterols (3). When these sterols accumulate within cells, the rate of proteolysis at Site-1 declines markedly. Cleavage at Site-2 also declines because this cleavage requires prior cleavage at Site-1 (3). As a result, the amounts of nuclear SREBP decline, and transcription of the target genes falls. The net effect is to prevent overaccumulation of cholesterol and fatty acids when intracellular sterol levels are already high.

Three isoforms of SREBP are known (5, 13, 14). SREBP-1a and 1c are derived from a single gene through use of alternate promoters that encode alternate first exons (5, 13, 14). SREBP-1a is much more active than SREBP-1c in stimulating transcription of all known target genes (15). The third protein, SREBP-2, is the product of a separate gene (6, 13), and it is also more active than SREBP-1c (15). In view of the regulatory role of the Site-1 protease, further knowledge of its structure and mode of regulation is desirable. A first step would be the identification of the precise site at which the Site-1 protease cuts SREBPs. In previous studies we have shown that this cleavage is abolished when arginine 519 in the luminal loop of SREBP-2 (or the corresponding arginine of SREBP-1a) is changed to alanine by in vitro mutagenesis (2). The size of the cleavage product, as determined by SDS-PAGE, is consistent with cleav-

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* The abbreviations used are: SREBP, sterol regulatory element-binding protein; ER, endoplasmic reticulum; LDL, low density lipoprotein; SCAP, SREBP cleavage-activating protein; PAGE, polyacrylamide gel electrophoresis; VAI, virus-associated I.

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age at or near this arginine (3).

In the current studies we have used a combination of in vitro mutagenesis, epitope tagging, immunoprecipitation, and biochemical sequencing to determine the precise location of Site-1 in SREBP-2. We found, surprisingly, that cleavage does not occur at arginine 519, but rather it occurs 3 residues further toward the COOH terminus, namely at leucine 522. Arginine 519 seems to be the NH₂-terminal residue in a tetrapeptide sequence, RXXL, that serves as a recognition signal for the Site-1 protease.

EXPERIMENTAL PROCEDURES

Materials—We obtained HSV-Tag™ and T7-Tag™ monoclonal antibodies from Novagen; v-H-Ras(Add-1)-agarose linked monoclonal antibody was obtained from Oncogene. Protein G-Sepharose⁴ was Fast Flow beads were obtained from Pharmacia Biotech Inc., t-(⁹⁸Sm)ethionine (>1000 Ci/mol) was obtained from DuPont NEN, and glycosidases were obtained from New England Biolabs.

Construction of pTK-HSV-BP2-Ras-T7—pTK-HSV-BP2-Ras-T7 encodes an epitope-tagged SREBP-2/Ras fusion protein (1391 amino acids) consisting of an initiator methionine, two tandem copies of the HSV epitope (QELAPAVEDPED), six novel amino acids (IDGTVP) encoded by a site that consists of restriction sites for BspDI and a human SREBP-2 (amino acids 14–1141), two novel amino acids (HM) encoded by the sequence for restriction site NdeI, human H-Ras (amino acids 2–189), and three tandem copies of the T7 epitope (HMASMTGQQM- GAAAAMASMTGQQMGPASMTGQQMQGMLINM).

pTK-HSV-BP2-Ras-T7 was constructed from a previously described plasmid, pTK-HSV-BP2-T7 (16), by insertion of a cDNA segment encoding human H-Ras between the sequences for SREBP-2 and the first copy of the T7 epitope. The nucleotide sequence encoding amino acids 2–189 of human H-Ras was obtained by polymerase chain reaction on pRcCMV-H-Ras (17) with a pair of primers containing an NdeI site at each 5’ end using Pfu DNA polymerase. The amplified product was digested with NdeI and cloned into the unique NdeI site between human SREBP-2 and the three tandem copies of the T7 epitope. Two independent clones were used in each of the transfections.

Construction of pTK-HSV-BP2/LDLRTM—pTK-HSV-BP2/LDLRTM encodes an epitope-tagged SREBP-2 fusion protein in which a 26-amino acid region that includes the second transmembrane domain of the human LDL receptor (amino acids 708–729) is replaced by a novel amino acid sequence containing 157-amino acid chimeric protein consisting of an initiator methionine, two tandem copies of the HSV epitope, six novel amino acids (IDGTVP), human SREBP-2 (amino acids 14–534), two novel amino acids (TG), human LDL receptor (amino acids 708–729), two novel amino acids (SG), and human SREBP-2 (amino acids 561–1141).

Plasmid pTK-HSV-BP2/R519A/LDLRTM is identical to pTK-HSV-BP2/LDLRTM except for the R519A point mutation in the luminal loop of the SREBP-2 sequence. This plasmid was constructed by site-directed mutagenesis.

Construction of pTK-HSV-BP2-NGT and pTK-HSV-BP2-NSS/NGT—pTK-HSV-BP2-NGT and pTK-HSV-BP2-NSS/NGT encode epitope-tagged SREBP-2 fusion proteins in which serine 515 in the loop region of SREBP-2 is replaced by a novel amino acid sequence containing one or two N-linked glycosylation sites, either NSS/G or NSSGSS/NGNT, respectively. Both plasmids were constructed by site-directed mutagenesis.

Site-directed Mutagenesis—Oligonucleotide site-directed mutagenesis was carried out using single-stranded, uracil-containing DNA (19) using the Mutagen Phagemid In Vitro Mutagenesis Version-2 kit (Bio-Rad) (2). The mutations were confirmed by sequencing the relevant region, and at least two independent clones of each mutant were independently transfected.

Cell Culture, Transfection, and Cell Fractionation—Monolayers of human embryonic kidney 293 cells were set up on day 0 (4 x 10⁵ cells/100-mm dish) and cultured in 8–9% CO₂, 95° C in medium A (Dulbecco's modified Eagle's medium containing 100 units/ml penicillin and 100 µg/ml streptomycin) supplemented with 10% (v/v) fetal calf serum (4). On day 2, cells were transfected with 4 µg of pTK empty vector (mock) or the indicated plasmid as described (2). Three h after transfection, the cells were switched to medium B (medium A containing 10% newborn calf lipoprotein-deficient serum, 50 µM compactin, and 50 µM sodium mevalonate) in the absence of neomycin as indicated in the legends. After incubation for 20 h, the cells received N-acetyl-leucinal-leucinal-norleucinal at a final concentration of 25 µM (2), and the cells were harvested 3 h later (2). The pooled cell suspension from 2 dishes was allowed to swell in hypotonic buffer (4) for 30 min at 0°C, passed through a 22.5-gauge needle 30 times, and centrifuged at 1000 x g at 4°C for 7 min. The 1000 x g pellet was resuspended in 0.1 ml of buffer C (10 mM Hepes-KOH (pH 7.4), 0.42 mM NaCl, 2.5% (v/v) glycerol, 1.5 mM MgCl₂, 0.5 mM sodium EDTA, 0.5 mM sodium EGTA, 1 mM dithiothreitol, and a mixture of protease inhibitors (4)). The suspension was rotated at 4°C for 1 h and centrifuged at top speed in a microfuge for 15 min at 4°C. The supernatant is designated nuclear extract. The supernatant from the original 1000 x g spin was centrifuged at 10⁵ x g for 30 min at 4°C in a Beckman TLS 100.2 rotor, and the pellet was dissolved in 0.1 ml of SDS lysis buffer (1) and designated membrane fraction.

Glycosidase Sensitivity of Membrane-bound SREBP-2—Glycosidase sensitivity of SREBP-2 was carried out as described (4). Monolayers of 293 cells were set up on day 0 (4 x 10⁵ cells/60-mm dish) and cultured as described above. On day 2, the cells were transfected with 7 µg of pTK empty vector (mock), pTK-HSV-BP2-NGT, and pTK-HSV-BP2- NSS/NGT, respectively. Three h after transfection, the cells were switched to medium B in the presence of sterols as described in Fig. 1. After incubation for 20 h, the cells were harvested, and the pooled cell suspension from 4 dishes was fractionated. The 10⁵ x g membrane pellet was washed once with buffer A (4) and resuspended in 180 µl of buffer A containing 1% (v/v) Triton X-100 without protease inhibitors. Aliquots of the 10⁵ x g membrane fraction (0.16 mg in 40 µl of buffer A) were boiled for 5 min in the presence (peptide N-glycosidase F and endoglycosidase H reactions) or in the absence (neuraminidase reactions) of 0.5% (v/v) SDS and 1% (v/v) β-mercaptoethanol for 5 min, after which the indicated amount of glycosidase was added and incubated at 37°C for 2 h as described in Fig. 10.

Cell lysate analysis—Samples of the nuclear extract and the 10⁵ x g membrane fraction were mixed with 5× SDS loading buffer (20). Protein concentration was measured with a BCA kit (Pierce). After SDS-PAGE in 8% gels, proteins were transferred to Hybond-C extra nitrocellulose membranes (Amersham Corp.). Immunoblot analysis was carried out with a horseradish peroxidase detection kit using the SuperSignal™ CL-HRP Substrate System according to the manufacturer’s instructions except that nitrocellulose membranes were blocked in phosphate-buffered saline containing 0.05% (v/v) Tween 20, 5% (v/v) nonfat dry milk, and 5% (v/v) heat-inactivated newborn calf serum. The chimeric proteins were visualized with 0.5 µg/ml HSV-Tag™ monoclonal antibody or with 10 µg/ml IgG-1C6, a mouse monoclonal antibody directed against amino acids 533–1141 of human SREBP-2 (4). Gels were calibrated with prestained molecular weight markers. Filters were exposed at room temperature to Reflection™ NEF-496 film (DuPont NEN).

Metabolic Labeling and Immunoprecipitation of Epitope-tagged SREBP-2/Ras Fusion Protein and Its COOH-terminal Fragment—Monolayers of 293 cells were set up on day 0 (7 x 10⁵ cells/60-mm dish) and cultured as described above. On day 1, the cells were transfected with 4 µg of the wild-type or mutan version of pTK-HSV-BP2-Ras-T7, 1 µg of pCMV-SCAP/D4343N (16), and 2 µg of pVA1 as described above. pVA1 encodes the adenovirus virus-associated 1 RNA gene, which enhances translation of transfected CNAs (21). The cells were incubated for 20 h with 50 µM compactin and 50 µM sodium mevalonate, at which time the medium was changed to 1.3 mM of mevinolin-free Dulbecco’s modified Eagle’s medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 0.5% newborn calf serum, 50 µM compactin, and 50 µM sodium mevalonate. After incubation for 1 h at 37°C, 25 µg/ml N-acetyl-leucinal-leucinal-norleucinal was added, and the cells were pulse-labeled with 700 µCi/ml of [⁹⁸Sm]ethionine for 6 h at 37°C. The cells from four dishes were harvested and pooled, and the membrane fraction was prepared as described above.

The pooled membrane fraction from the four dishes was resuspended...
in 0.1 ml of SDS lysis buffer at room temperature. All subsequent operations were carried out at 4 °C unless otherwise stated. The suspension was rotated for 30 min in 5 ml of buffer D (50 mM Tris-Cl (pH 7.5), 125 mM NaCl, 0.5% (v/v) SDS, 1.25% (v/v) Triton X-100, 1.25% (v/v) deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μM/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 25 μg/ml N-acetyl-leucinal-leucinal- norleucinal, and 1 mM diithiothreitol), after which 20 μl of preimmune whole rabbit serum, 40 μg of irrelevant mouse monoclonal antibody IgG-2001 (22), and 0.2 ml of Protein G-Sepharose beads were added. After rotation for 16 h, the mixture was centrifuged at 1000 × g for 7 min. The resulting supernatant was mixed with an additional 20 μl of preimmune whole rabbit serum, 40 μg of irrelevant monoclonal antibody IgG-2001, and 0.2 ml of Protein G-Sepharose beads, rotated for 2 h, and centrifuged at 1000 × g for 3–7 min. To the supernatant were added 1 μg of T7-Tag monoclonal antibody, 25 μg of υ-H-Ras(Ab-1)-agarse linked monoclonal antibody, and 20 μg of anti-COOH-terminal SREBP-2 monoclonal antibody IgG-1C6. After rotation for 2.5 h, 50 μl of Protein G-Sepharose beads were added, followed by rotation for 2.5 h and centrifugation at 1000 × g for 3–7 min. The beads were washed once by rotation with buffer D for 16 h, followed by four washes in buffer D for 1 h each. The washed beads were resuspended in 0.1 ml of 2× SDS loading buffer (20) containing 10% (v/v) β-mercaptoethanol and boiled for 5 min. After centrifugation at 1000 × g at room temperature for 3 min, the supernatant was transferred to a fresh tube (tube A). The beads were re-eluted with 40 μl of 5× SDS loading buffer containing 25% β-mercaptoethanol and boiled for 5 min. After centrifugation at 1000 × g at room temperature for 3 min, the supernatant was transferred to tube A, and the entire volume was boiled again for 5 min before SDS-PAGE.

RESULTS AND DISCUSSION

To identify the exact position of Site-1, we prepared a cDNA (pTK-HSV-BP2-Ras-T7) encoding a triply tagged version of SREBP-2, which we designate SREBP-2/Ras (Fig. 1). The NH₂-terminal sequence analysis of 35S-labeled COOH-terminal Fragment of Epitope-tagged SREBP-2 Fusion Protein—Aliquots of the immunoprecipitated samples (from 1.3 dishes of cells) were subjected to SDS-PAGE on 8% gels and transferred to polyvinylidene fluoride membranes (Immobilon-P®, Millipore). After drying, the membranes were exposed to an imaging plate and scanned in a Fuji X Ras 1000 phosphorimager. The band containing the COOH-terminal product of the cleavage reaction (M₀, ~83,000) was excised and subjected directly to multiple cycles of Edman degradation on an Applied Biosystems model 477A sequencer. Fractions from each cycle (148 μl) were collected and counted in a scintillation counter.

To demonstrate that SREBP-2/Ras is cleaved at Site-1 in a physiologic fashion, we transfected 293 cells with a vector encoding this construct and another encoding a mutated version in which arginine 519 was changed to alanine (Fig. 1). We used the relatively weak thymidine kinase promoter, which produces near physiological levels of this protein (2). Cells were incubated in inducing medium that contains the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor compactin to block cholesterol synthesis plus a low concentration of mevalonate to promote sterol end product synthesis. The medium was either devoid of sterols (− sterols) or it was supplemented with a mixture of 25-hydroxycholesterol and cholesterol (+ sterols), which suppresses cleavage at Site-1 (3). Cell membranes were isolated, solubilized with SDS, subjected to SDS-PAGE, and immunoblotted with IgG-1C6, a monoclonal antibody directed against the COOH terminus of SREBP-2 (4).

When cells were transfected with the cDNA encoding the SREBP-2/Ras chimeric protein, the membranes contained the 83-kDa COOH-terminal fragment, which was the product of cleavage at Site-1 (Fig. 1, lane 1, transfected C). The amount of this fragment was reduced in the presence of sterols (lane 4). The IgG-1C6 antibody also visualized the 62-kDa COOH-terminal fragment of SREBP-2/Ras (lane 1, transfected C), and this was also decreased by sterols (lane 4). To test the physiologic relevance of the observed cleavage of SREBP-2/Ras, we cotransfected a cDNA encoding the D443N mutant version of SREBP-2 with a cDNA encoding the D443N mutant version of SREBP-2, a protein that was previously shown to render the COOH-terminal fragment of endogenous SREBP-2 (lane 3, endogenous C), and this was also decreased by sterols (lane 4). To test the physiologic relevance of the observed cleavage of SREBP-2/Ras, we cotransfected a cDNA encoding the D443N mutant version of SREBP-2, a protein that was previously shown to render the COOH-terminal fragment of endogenous SREBP-2 (lane 3, endogenous C), and this was also decreased by sterols (lane 4). To test the physiologic relevance of the observed cleavage of SREBP-2/Ras, we cotransfected a cDNA encoding the D443N mutant version of SREBP-2, a protein that was previously shown to render the COOH-terminal fragment of endogenous SREBP-2 (lane 3, endogenous C), and this was also decreased by sterols (lane 4). To test the physiologic relevance of the observed cleavage of SREBP-2/Ras, we cotransfected a cDNA encoding the D443N mutant version of SREBP-2, a protein that was previously shown to render the COOH-terminal fragment of endogenous SREBP-2 (lane 3, endogenous C), and this was also decreased by sterols (lane 4). To test the physiologic relevance of the observed cleavage of SREBP-2/Ras, we cotransfected a cDNA encoding the D443N mutant version of SREBP-2, a protein that was previously shown to render the COOH-terminal fragment of endogenous SREBP-2 (lane 3, endogenous C), and this was also decreased by sterols (lane 4). To test the physiologic relevance of the observed cleavage of SREBP-2/Ras, we cotransfected a cDNA encoding the D443N mutant version of SREBP-2, a protein that was previously shown to render the COOH-terminal fragment of endogenous SREBP-2 (lane 3, endogenous C), and this was also decreased by sterols (lane 4). To test the physiologic relevance of the observed cleavage of SREBP-2/Ras, we cotransfected a cDNA encoding the D443N mutant version of SREBP-2, a protein that was previously shown to render the COOH-terminal fragment of endogenous SREBP-2 (lane 3, endogenous C), and this was also decreased by sterols (lane 4). To test the physiologic relevance of the observed cleavage of SREBP-2/Ras, we cotransfected a cDNA encoding the D443N mutant version of SREBP-2, a protein that was previously shown to render the COOH-terminal fragment of endogenous SREBP-2 (lane 3, endogenous C), and this was also decreased by sterols (lane 4). To test the physiologic relevance of the observed cleavage of SREBP-2/Ras, we cotransfected a cDNA encoding the D443N mutant version of SREBP-2, a protein that was previously shown to render the COOH-terminal fragment of endogenous SREBP-2 (lane 3, endogenous C), and this was also decreased by sterols (lane 4).
Identification of a Sterol-regulated Cleavage Site in SREBP-2

To determine whether the COOH-terminal fragment could be labeled with [35S]methionine and immunoprecipitated, we transfected 293 cells with a cDNA encoding the SREBP-2/Ras chimera and labeled the cells with [35S]methionine. A membrane fraction was prepared, solubilized with detergents, and immunoprecipitated with a mixture of three antibodies directed against H-Ras, the T7 epitope tag, and the COOH-terminal segment of SREBP-2. The immunoprecipitate was subjected to SDS-PAGE and phosphorimager analysis, which revealed a band of ~83 kDa that was absent from mock-transfected cells (Fig. 3A). Immunoblot analysis of the precipitate (Fig. 3B) and the supernatant (data not shown) confirmed that all of the COOH-terminal fragment was precipitated. In future experiments, the 83-kDa radiolabeled band was excised from the nitrocellulose membrane and used for radiochemical sequencing.

Fig. 4 shows a series of experiments in which 293 cells were transfected with cDNAs encoding the SREBP-2/Ras chimera with the wild-type sequence in the luminal loop or with the three methionine substitutions at positions 525, 526, and 529. The cells were then incubated with [35S]methionine. After immunoprecipitation and electrophoresis, the COOH-terminal fragments were eluted and subjected to automated Edman sequencing. The radioactivity released in each cycle was determined. The protein with the wild-type sequence in the lumenal loop or with the wild-type sequence in the COOH-terminal domain of SREBP-2 (B) showed a clear peak of 35S radioactivity in cycle 3 from Edman degradation (Fig. 4B). The S526M and G529M mutants showed peaks at cycles 4 and 7, respectively (Fig. 4C and D). These findings indicate strongly that the COOH-terminal fragment begins with serine 523 and that Site-1 cleavage occurs between this residue and unknown.
Identification of a Sterol-regulated Cleavage Site in SREBP-2

We examined the NH\(_2\)-terminal region of COOH-terminal fragment of SREBP-2 after cleavage of transfected epitope-tagged SREBP-2/Ras fusion protein. 293 cells were set up for experiments and transfected with the wild-type (A) or the indicated methionine mutant version of pTK-HSV-BP2-Ras-T7 (B–D) plus 2 \( \mu \)g of pVAI and 1 \( \mu \)g of pCMV-SCAP(D443N). On day 2, the cells were radiolabeled with \[^{35}S\]methionine, harvested, and fractionated, after which the cleaved COOH-terminal fragment of human SREBP-2 was immunoprecipitated. Aliquots of the immunoprecipitated samples (from 1.3 dishes of cells) were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were exposed to an imaging plate and scanned in a Fuji X Bas 1000 phosphorimager. The band containing the COOH-terminal product of the cleavage reaction was excised and subjected to multiple cycles of Edman degradation. The radioactivity recovered at each cycle of Edman degradation is plotted. Amino acid sequences shown at the top of each panel correspond to the SREBP-2 sequence in the region of the postulated site of sterol-regulated cleavage.

To determine the amino acid residues that are important for cleavage of SREBP-2 at Site-1, we made a systematic series of mutations in which alanine was individually substituted for leucine 522 (indicated by the arrows in the amino acid sequences of Fig. 4).

Fig. 5 shows illustrative results from one of the alanine scanning experiments. When the cDNA encoded the wild-type SREBP-2 sequence, we observed the mature NH\(_2\)-terminal fragment of SREBP-2 in nuclear extracts (Fig. 5, lane 3). This fragment was abolished in the presence of sterols (lane 4). The R519A mutant was not cleaved (lane 5), but the D510A and Q511A mutants were cleaved as efficiently as the wild-type protein (lanes 7 and 9, respectively).

Fig. 6 summarizes the results of all 27 alanine scanning experiments. At the top we show the sequences of the lumenal loops of four of the SREBPs that have been sequenced, two from the human (5, 6) and two from the hamster (24, 25). Conserved residues are highlighted. Only two single alanine substitutions reduced cleavage dramatically: the R519A and the L522A mutations (Mutants No. 11 and 14, respectively). Three of the multiple alanine replacements reduced cleavage (Mutant No. 21, 24, and 25). All of these mutations included the L522A substitution. The other multiple alanine replacements did not reduce cleavage. These no-effect mutations included the change of Arg\(^{159}\)-Ser-Val-Leu\(^{222}\) to Arg\(^{159}\)-Ala-Ala-Leu\(^{222}\) (Mutant No. 23). Cleavage was also not affected when the serine following the cleavage site was changed to alanine (S523A, Mutant No. 15). These data indicate that arginine 519 at the P4 position and leucine 522 at the P1 position are the only 2 residues in the region of Site-1 that cannot tolerate alanine substitutions.

To further dissect the requirement for arginine 519, we systematically replaced this residue with 12 different residues (Fig. 7). When lysine was substituted for this arginine, only a partial loss of cleavage activity was observed. Two negatively charged residues (glutamic acid and aspartic acid) permitted a barely detectable level of cleavage. All other substitutions abolished cleavage. The location of arginine 519 was critical. Cleavage was abolished when we replaced arginine 519 with alanine and then inserted a new arginine at positions 517, 520, or 521. These mutations effectively moved the position of the arginine either toward the NH\(_2\) terminus or COOH terminus.

We also tested the specificity of the requirement for leucine 522 at the cleavage site (Fig. 8). Cleavage was markedly reduced but not totally abolished when this residue was changed to arginine, alanine, or phenylalanine. It was abolished when leucine 522 was changed to glutamic acid or valine. The residue at the P'1 position was not critical. This residue is serine in human and hamster SREBP-2, but it is glutamic acid and...
glycine in hamster and human SREBP-1, respectively. Replacement of serine 523 in human SREBP-2 with glutamic acid or glycine preserved normal cleavage. The cleavage was also unaffected when this residue was changed to arginine, alanine, leucine, or phenylalanine. A moderate reduction was observed when it was changed to cysteine.

To determine whether the second transmembrane domain contributes to recognition by the Site-1 protease, we replaced this sequence with the membrane-spanning region of another protein, namely, the LDL receptor (Fig. 9). The LDL receptor is a type 1 transmembrane protein of the plasma membrane with a single membrane-spanning segment oriented with its NH2 terminus in the extracellular space and its COOH terminus in the cytosol (18). This orientation is the same as the orientation of the second transmembrane domain of SREBP-2 (4). The chimeric protein containing the LDL receptor transmembrane domain was cleaved as efficiently as the wild-type protein, as judged by the amount of NH2-terminal fragment found in the nucleus (Fig. 9, lane 5). Cleavage was abolished by sterols (lane 6). It was also abolished when arginine 519 of the chimera was changed to alanine (lane 7). These data indicate that the precise sequence of the second transmembrane domain is not important for cleavage at Site-1 (or Site-2). A hydrophobic sequence at this position is required, however. When the second transmembrane domain was deleted without replacement by another transmembrane domain (HSV-SREBP-2 Δ535–560), cleavage at Site-1 and -2 was abolished (data not shown).

Previously we presented evidence that the lumenal loop sequence is indeed in the ER lumen (4). We were forced to insert a long epitope-containing peptide because shorter epitopes were not recognized by antibodies after insertion into the lumenal loop of SREBP-2. Sealed membrane vesicles were prepared from cells expressing this protein, and the segment containing the epitope was shown to be protected from digestion by trypsin in the absence, but not the presence, of detergents. The epitope-containing segment was also demonstrated to undergo N-linked glycosylation. The problem with these studies is that the chimeric protein was not cleaved by the Site-1 protease. Therefore, we could not be certain that the long epitope-containing peptide had not altered the orientation of the protein.

To circumvent this problem, we took advantage of the observation that the precise sequence on the NH2-terminal side of arginine 519 is not essential for Site-1 cleavage. Therefore, we prepared a plasmid encoding forms of SREBP-2 in which we inserted either 3 or 9 amino acids into this sequence in place of serine 515 (Fig. 10A). The inserted amino acids included either one or two sites for N-linked glycosylation (Asn-Xaa-Ser/Thr).

After transfection, membrane pellets containing the SREBP-2 inserted into the luminal loop (4). We were forced to insert a long protein segment because shorter epitopes were not recognized by antibodies after insertion into the luminal loop of SREBP-2. Sealed membrane vesicles were prepared from cells expressing this protein, and the segment containing the epitope was shown to be protected from digestion by trypsin in the absence, but not the presence, of detergents. The epitope-containing segment was also demonstrated to undergo N-linked glycosylation. The problem with these studies is that the chimeric protein was not cleaved by the Site-1 protease. Therefore, we could not be certain that the long epitope-containing peptide had not altered the orientation of the protein.

To circumvent this problem, we took advantage of the observation that the precise sequence on the NH2-terminal side of arginine 519 is not essential for Site-1 cleavage. Therefore, we prepared a plasmid encoding forms of SREBP-2 in which we inserted either 3 or 9 amino acids into this sequence in place of serine 515 (Fig. 10A). The inserted amino acids included either one or two sites for N-linked glycosylation (Asn-Xaa-Ser/Thr). After transfection, membrane pellets containing the SREBP-2...
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Fig. 9. Sterol-regulated cleavage of epitope-tagged SREBP-2/LDLRtm chimeric protein in transfected 293 cells. A, schematic diagram of the chimeric protein encoded by pTK-HSV-BP2/LDLRtm, showing the replacement of the second transmembrane domain of human SREBP-2 with the transmembrane domain of the LDL receptor. B, 293 cells were set up for experiments, transfected with 4 μg of either wild-type pTK-HSV-BP2 or the indicated mutant plasmid, incubated in the absence or presence of sterols, and fractionated as described in the legend of Fig. 1. Aliquots of the nuclear extracts (60 μg of protein) and membranes (80 μg) were subjected to SDS-PAGE and immunoblot analysis with 0.5 μg/ml HSV-Tag™ antibody. The filters were exposed to film for 7 s. M and P denote the cleaved NH2-terminal mature and uncleaved precursor forms of SREBP-2, respectively. The other bands are present in mock-transfected cells and represent proteins that cross-react with the anti-HSV tag antibody.

A precursor was digested with glycosidases, and the change in mobility on SDS-PAGE was determined by immunoblotting. As shown in Fig. 10B, the mobility of the SREBP-2 precursors with either one or two glycosylation sites was increased by treatment with peptide N-glycosidase F (lanes 2 and 7) and by endoglycosidase H (lanes 3 and 8), but not by neuraminidase (lanes 4 and 9). The changes were greater for the construct with two glycosylation sites. This pattern indicates that the lumenal loop sequence contains N-linked carbohydrates of the high mannose type. The lack of processing to an endoglycosidase H-resistant form indicates that the precursor is located in the ER and not the Golgi complex. Fig. 10C shows immunoblots of nuclear extracts and membrane pellets from cells expressing SREBP-2 with the wild-type lumenal sequence, the R519A mutation, or the insertion of one or two N-linked glycosylation sites. The cells were incubated under conditions that induce SREBP-2 cleavage (− sterols) or suppress cleavage (+ sterols). The membrane-bound precursor form with one glycosylation site (lanes 7 and 8) migrated slower than the wild-type protein (lanes 3 and 4), and the form with two glycosylation sites migrated even slower (lanes 9 and 10). Under inducing conditions the nuclear extracts contained the NH2-terminal fragments of all proteins except the R519A mutant. Cleavage of all proteins was suppressed by sterols. This experiment demonstrates that the glycosylated precursor of SREBP-2 remain susceptible to sterol-regulated cleavage at Site-1 and subsequent cleavage at Site-2. The presence of sterols inhibited the carbohydrate chains did, however, impede cleavage. The absolute amount of cleavage was reduced by ~50% for the construct containing one glycosylation site and by ~80–90% for the protein containing two glycosylation sites.

Considered together, the data in this paper indicate that the Site-1 protease cleaves the peptide bond between leucine 522 and serine 523 in the lumenal loop of SREBP-2. The only residues that seem to be crucial for recognition are arginine 519 and serine 523.
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and leucine 522. The location of the arginine relative to the leucine also seems to be crucial. The recognition sequence seems to be RXXL where X can be serine, valine, or alanine, at least. The RXXL sequence is conserved in the four hamster and human SREBPs shown in Fig. 6 and also in SREBP-1c/ADD1 from the rat (RSMLE) (26) and SREBP/HLH106 from Drosophila (RRILS) (27). We believe that other features of the luminal loop are also crucial for cleavage because moving the RXXL sequence to other sites in the luminal loop substantially reduced cleavage (data not shown).

Although the current studies identify the site within SREBP-2 that is cleaved by the Site-1 protease, they do not reveal where in the cell this cleavage occurs. Immunofluorescence (24) and cell fractionation studies\(^2\) have shown that SREBPs are initially found on membranes of the nuclear envelope and ER. We do not yet know whether the Site-1 protease operates in these organelles or whether the SREBPs must be transported to some other site where cleavage takes place.

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\(^2\) J. Sakai, E. A. Duncan, M. S. Brown, and J. L. Goldstein, unpublished observations.
Cleavage Site for Sterol-regulated Protease Localized to a Leu-Ser Bond in the Lumenal Loop of Sterol Regulatory Element-binding Protein-2

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