Second-site Cleavage in Sterol Regulatory Element-binding Protein Occurs at Transmembrane Junction as Determined by Cysteine Panning*

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In response to sterol deprivation, two sequential proteolytic cleavages release the NH₂-terminal fragments of sterol regulatory element-binding proteins (SREBPs) from cell membranes. The fragments translocate to the nucleus where they activate genes involved in cholesterol and fatty acid metabolism. The SREBPs are bound to membranes in a hairpin fashion. The NH₂-terminal and COOH-terminal domains face the cytoplasm, separated by two membrane spanning segments and a short lumenal loop. The first cleavage occurs at Site-1 in the lumenal loop. The NH₂-terminal fragment is then released by cleavage at Site-2, which is believed to lie within the first transmembrane segment. Here, we use a novel cysteine panning method to identify the second cleavage site (Site-2) in human SREBP-2 as the Leu⁴⁸⁴-Cys⁴⁸⁵ bond that lies at the junction between the cytoplasmic NH₂-terminal fragment and the first transmembrane segment. We transfected cells with cDNAs encoding fusion proteins with single cysteine residues at positions to the NH₂-terminal and COOH-terminal sides of cysteine 485. The NH₂-terminal fragments were tested for susceptibility to modification with N'-[2-hydroxy-1,1-bis(2-hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis.

Cells maintain cholesterol homeostasis through a feedback pathway that depends upon the regulated proteolytic processing of a pair of membrane-bound transcription factors, sterol regulatory element-binding proteins-1 and -2 (SREBP-1 and SREBP-2)¹ (reviewed in Ref. 1). Cells deprived of cholesterol process the SREBPs proteolytically to release the NH₂-terminal domains which travel to the nucleus to activate transcription of genes that regulate several metabolic pathways: uptake of cholesterol and fatty acids (low density lipoprotein receptor and lipoprotein lipase) (1, 2), synthesis of cholesterol (3-hydroxy-3-methylglutaryl-CoA reductase, 3-hydroxy-3-methylglutaryl-CoA synthase, farnesyl diphosphate synthase, squalenone synthase) (1, 3–5), synthesis of fatty acids (acetyl-CoA carboxylase, fatty acid synthase, stearoyl-CoA desaturase) (1, 2, 6, 7), and synthesis of triglycerides (glycerol-3-phosphate acyltransferase) (8). When cells accumulate sterols, proteolysis of the SREBPs is suppressed, nuclear SREBPs decline, and the transcription of the target genes is reduced. This regulation assures a steady supply of cholesterol and fatty acids while preventing their overaccumulation.

SREBPs are unique among transcription factors because they are synthesized as membrane-bound precursors with three domains. The precursors are bound to the endoplasmic reticulum and nuclear envelope in a hairpin orientation (9). The cytoplasmic NH₂-terminal domain (−480 amino acids) contains an acidic transcriptional activation sequence and a basic helix-loop-helix leucine zipper element that is responsible for DNA binding and dimerization. The middle segment (−80 amino acids) consists of two-membrane spanning helices separated by a short hydrophilic loop of 31 amino acids that projects into the lumen of the endoplasmic reticulum and nuclear envelope. The COOH-terminal segment (−590 amino acids) projects into the cytoplasm. It is designated the regulatory domain because it is necessary for the sterol-regulated proteolysis of the SREBPs (10, 11).

In response to sterol deprivation, the NH₂-terminal domains of the SREBPs are released from membranes by a two-step proteolytic cascade (1, 12). The first proteolytic cleavage is regulated by the putative sterol sensor, SCAP (SREBP cleavage-activating protein) (13), which forms a complex with the COOH-terminal regulatory domain of the SREBPs (10, 11). The complex triggers the first cleavage, which occurs at Site-1 in the middle of the lumenal loop (14). This separates the NH₂-terminal half of the SREBP from the COOH-terminal half, but both halves remain membrane bound. The membrane-bound NH₂-terminal fragment of SREBP is termed the intermediate (12). Next, a second protease, designated the Site-2 protease (S2P), cleaves the NH₂-terminal intermediate, apparently within the first transmembrane segment (12, 15). The cleaved NH₂-terminal domain now travels to the nucleus where it activates transcription by binding to sterol regulatory elements in the promoters of the genes described above. The activity of S2P is not itself regulated by sterols, but it cleaves the NH₂-terminal domain at Site-2 only after the protein has been cleaved at Site-1 (12, 15).

The cDNA encoding S2P, a multiple membrane-spanning protein that is required for Site-2 proteolysis, was recently identified by complementation cloning in a mutant line of Chinese hamster ovary cells (M19 cells) (16) that lack this activity.
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(12, 15). Lacking the ability to cleave the SREBPs at Site-2, M19 cells are unable to transcribe cholesterol biosynthetic genes and are thus cholesterol auxotrophs (12, 15, 16). When transfected into M19 cells, the S2P cDNA restores cleavage at Site-2 and relieves the need for exogenous cholesterol. S2P contains the consensus metalloprotease zinc-binding site, HEXXH (15). Mutation of this site abolishes S2P activity, and it was concluded that S2P is indeed the Site-2 protease (15).

We recently mapped Site-1 of human SREBP-2 to the Leu522, Ser523 bond in the lumenal loop by a combination of mutagenesis, [35S]methionine labeling, and radiochemical sequencing of the cleaved COOH-terminal fragment (14). To estimate the location of Site-2, we previously replaced the NH2 terminus of SREBP-2 with H-Ras and used truncated forms of this fusion protein as size standards for comparison with the fully processed fusion protein (12). These studies suggested that Site-2 is located somewhere within the first transmembrane segment, but the precise peptide bond was not identified. Proteolysis of the SREBPs at Site-2 is abolished when the sequence D478RSR, which is immediately proximal to the first transmembrane segment, is replaced with AS (12).

In designing a strategy to identify the precise location of Site-2, we faced several obstacles. Radiochemical sequencing was not possible because this technique relies upon the ability to isolate the COOH-terminal product of the cleavage reaction (14). Following Site-2 proteolysis, this small fragment (~40 amino acids) is likely to be rapidly degraded. Mass spectrometry could be used to determine the precise mass of the nuclear NH2-terminal fragment. However, this would require the isolation of relatively large amounts of this fragment from cell nuclei. This is not feasible in cultured cells because overexpression of SREBPs by transfection leads to artifactual proteolytic processing (17). Faithful processing is observed only when the cells express low levels of SREBPs through the use of weak promoters, such as the herpes simplex virus (HSV) thymidine kinase promoter (17).

We herein report a novel technique that permits the identification of the precise peptide bond cleaved during proteolysis at Site-2. We produced a fusion protein whose NH2-terminal fragment lacks cysteine. Each residue near Site-2 was singly replaced with cysteine, and the released NH2-terminal fragment lacks cysteine. Each residue near Site-2 was singly replaced with cysteine, and the released NH2-terminal fragment was isolated, and its susceptibility to a cysteine modification was tested. The isolated proteolytic product was digested with EcoRI and AgeI sites immediately following the H-Ras sequence in pTK-HSV-RasBP2(473–1141). The cDNA encoding amino acids 2–87 of rat ACBP was obtained by polymerase chain reaction using rat brain cDNA as a template (CLONTECH), an NH2-terminal primer flanked by an EcoRV site, and a COOH-terminal primer flanked by an AgeI site. The amplified product was digested with EcoRV and AgeI and ligated into the unique EcoRV and AgeI sites of the intermediate plasmid (described above).

The expression vector pTK-HSV-8His-ACBP/BP2 encodes the sequence described above with an insertion of 11 amino acids between the HSV tag and ACBP/BP2. To construct pTK-HSV-8His-ACBP/BP2, a pBR322 complementary oligonucleotides (top strand, 5′-ATCCATCACATCATCACATCACACCAAGG-3′; bottom strand, 5′-ACGGGTGTAGTGATGGATGGGAGGGATG-3′) were phosphorylated, annealed, and ligated into the unique EcoRI restriction site of pTK-HSV-BP2-ACBP/BP2. The inserted oligonucleotides encode 11 amino acids (isoleucine, 8 histidines, threonine, and glycine).

The expression vector pTK-HSV-8His-BP2 encodes the same sequence as pTK-HSV-BP2 (Ref. 17) except for the insertion of 10 amino acids (isoleucine, alanine, and 8 histidines) between the HSV tag and SREBP-2. To construct pTK-HSV-8His-BP2, complementary oligonucleotides (top strand, 5′-CCGACATCACATCATCACATCACATCATCATCATCATCATCATCATCATCATCATCAT-3′; bottom strand, 5′-CGATGTGATGGATGGATGGGATGAGGGATG-3′) were phosphorylated, annealed, and ligated into the unique BspDI restriction site of pTK-HSV-BP2. The structures of the above expression vectors were confirmed by sequencing all ligation junctions and the entire ACBP coding region.

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

Transfection of 293 Cells and Cell Fractionation—Monolayers of human embryonic kidney 293 cells were set up for experiments on day 0 (4 × 10^6 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 sulfate) supplemented with 10% (v/v) fetal calf serum. On day 2, the cells were transfected with 4 μg of pTK empty vector (mock) or the indicated plasmid using an MBS Transfection Kit (Stratagene) as described previously (9). 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) (9) in the absence or presence of sterols as indicated in the legends. Before transfections involving pH-HV-HSV-BP2 and its mutant versions, the cells received ALLN (N-acetyl-leucil-leucinal-norleucinal) at a final concentration of 25 μg/ml 3 h prior to harvest. Cells transfected with constructs encoding ACBP/SREBP-2 fusion proteins did not receive ALLN prior to harvest. Homogenates of cells were fractionated as described previously to yield three fractions: nuclear extract, membranes (105 g x g), and cytosol (105 g x g supernatant) (12).

Transfection of CHO-7 and M19 Cells and Cell Fractionation—Monolayers of CHO-7 and M19 cells (15) were set up for experiments on day 0 (7 × 10^6 cells/60-mm dish) in medium C (a 1:1 mixture of Ham’s F-12 medium and Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, 5% (v/v) fetal calf serum, 5 μg/ml cholesterol, 1 mM sodium mevalonate, and 20 μg/ml sodium olate). On day 1, cells were transfected with 6.5 μg of DNA/dish using LipofectAMINE® Reagent (Life Technologies) as described previously (15), except that all cells were grown in the absence of sterols and the cells did not receive ALLN. Cell fractionation was carried out as described previously (12) with the following modifications. The pooled cell suspension from two dishes was allowed to swell for 45 min at 0 °C in hypotonic buffer containing 50 mM Hepes-KOH (pH 7.4), 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, and 1 mM sodium EGTA, and protease inhibitors as described previously (9). Samples were passed through a 22.5-gauge needle 30 times, and centrifuged at 890 × g at 4 °C for 4 min. The supernatant was centrifuged at 105 × g for 30 min at 4 °C in a Beckman TLA 100.2 rotor, and the resulting pellet was dissolved in 0.1 ml of SDS lysis buffer (20) and designated the membrane fraction. The supernatant was centrifuged at 105 × g for 30 min at 4 °C in a Beckman TLA 100.2 rotor. The cytosol fraction was prepared by precipitating the final 105 × g supernatant with 5 volumes of acetone for 10 min at 0 °C, followed by centrifugation at top speed in a microcentrifuge at 4 °C for 15 min and resuspension of the pellet in 0.1 ml of SDS lysis buffer.

SDS-PAGE Immunoblot Analysis—Protein concentration was meas-
ured with a BCA kit (Pierce). Samples of nuclear extract, 10^5 × g membrane fraction, and cytosol were mixed with 5 × SDS loading buffer (21) and boiled for 5 min. SDS-PAGE was carried out using gels of 7, 12, or 15% acrylamide. Proteins were transferred to Hybond-C extra nitrocellulose membranes (Amer sham Corp.), and immunoblot analysis was performed for 15 min using 1% PIGG-ME (washed in buffer C) at 4°C. This pellet was resuspended in 100 μl of SDS lysis buffer plus 25 μl of 5 × SDS loading buffer. Samples were boiled for 5 min and subjected to SDS-PAGE immunoblot analysis as described above. The films were scanned with a Desk Scanner (Hewlett Packard, ScanJet 4cT), and quantification was performed on a Macintosh 7500 computer using the public domain NIH Image Program (developed at the U. S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

For identification of the Site-2 cleavage site in ACBP/SREBP-2 fusion proteins, 75 μl of washed Ni-NTA-agarose was added directly to 0.8 ml (2 mg of protein) of cytosol fractions from four pooled 60-mm dishes of transfected 293 cells. After rotation for 30 min at 4°C, the mixture was centrifuged at top speed in a microcentrifuge for 3 min at 4°C and further processed at room temperature through the steps of MBP derivatization, Ni-NTA-agarose chromatography, streptavidin precipitation, and immunoblot analysis as described above for SREBP-2, except that bovine serum albumin was substituted for cytochrome c in the streptavidin precipitation step.

RESULTS

We previously replaced the 472-amino acid NH2-terminal domain of human SREBP-2 with the 188-amino acid H-Ras protein and used prematurely terminated forms of this fusion protein as size standards for comparison with the NH2-terminal fragment generated from the fusion protein within cells (12). Immunoblot analysis after SDS-PAGE suggested that the Site-2 protease cleaves SREBP-2 somewhere within the ~20-kDa acidic sequence that comprises the first transmembrane segment (12). To increase the resolution of this technique, we sought a smaller protein that could replace the NH2-terminal segment of SREBP-2 while maintaining proper two-step processing. The acyl-CoA binding protein (ACBP) fits this criterion (Fig. 1A). ACBP is an 87-amino acid cytosolic protein (18). When fused to amino acids 473–1141 of human SREBP-2, the ACBP is predicted to produce a mature NH2-terminal segment of ~123 to 143 amino acids, which is one-fourth the size of the mature NH2-terminal segment of native SREBP-2.

To demonstrate that ACBP/SREBP-2 is cleaved through the normal two-step process, we performed a co-transfection experiment in wild-type CHO-7 cells and in M19 cells, a mutant line of CHO cells that lack S2P (12, 15). The cells were transiently transfected with a cDNA encoding ACBP/SREBP-2 tagged at its NH2-terminus with an epitope derived from the HSV envelope glycoprotein D (24) and driven by the HSV-thymidine kinase promoter. We also transfected cDNAs encoding pVAl, pSCAP(D443N), and either S2P or empty vector. pVAl encodes an RNA that stimulates translation of mRNAs produced from transfected cDNAs (23). It enhances expression of the ACBP/SREBP-2 fusion protein by severalfold in our experiments. pSCAP(D443N) encodes a mutant version of SCAP that was previously shown to increase the efficiency of cleavage of SREBPs at Site-1 (13). Membranes and cytosol were isolated from transfected cells and subjected to SDS-PAGE and immunoblot analysis with an antibody directed against the HSV epitope tag. The chimeric ACBP/SREBP-2 plasmid produced a full-length membrane-bound precursor with an apparent molecular mass of ~80 kDa (designated P in Fig. 1B, upper panel).

In the absence of co-transfection of S2P, the CHO-7 cells cleaved ACBP/SREBP-2, generating a mature form that was found in the cytosol and migrated at ~20 kDa on SDS-PAGE (designated M in lane 3). The M19 cells cleaved ACBP/SREBP-2 only at Site-1, generating an intermediate form of ~24 kDa that remained bound to membranes (designated I in lane 7). The M19 cells produced the cytosolic mature form when we co-transfected a plasmid encoding Myc-tagged S2P (designated M in...
FIG. 1. Site-2 protease-dependent cleavage of ACBP/SREBP-2 fusion protein. A, schematic comparison of the structures of wild-type SREBP-2 and the ACBP/SREBP-2 fusion protein encoded by pTK-HSV-ACBP/BP2, showing the relative sizes of the cytosolic NH$_2$-terminal domains. circles numbers 1 and 2 denote the two sequential sites of proteolytic cleavage. B, immunoblot analysis of the membrane and cytosolic fraction of CHO-7 and M19 cells transfected with pTK-HSV-ACBP/BP2. Cells were set up on day 0 and transfected on day 1 as described under “Experimental Procedures.” pTK empty vector (lanes 1, 2, 3, 5, and 6) or pTK-HSV-ACBP/BP2 (lanes 3, 4, 7, and 8) was co-transfected into cells (3 μg/dish) together with pVAl (1 μg/dish) and pCMV-SCAP(D443N) (0.5 μg/dish) in the absence or presence of pCMV-Myc-S2P (0.5 μg/dish) as indicated. The total amount of DNA was adjusted to 6.5 μg/dish by the addition of pcDNA3 empty vector. On day 2, the cells were harvested and fractionated as described under “Experimental Procedures.” Aliquots of the cytosol (100 μg of protein for lanes 1–3, 5, 7, and 9–11 and 1–5 μg for lanes 4, 6, and 8) were adjusted to 100 μg of total protein by the addition of mock-transfected cytosol. Protein samples were subjected to Tricine SDS-PAGE and immunoblot analysis with 0.5 μg/ml IgG-HSV-Tag as described under “Experimental Procedures.” The filter was exposed to film for 30 s. The migration of a 56-kDa irrelevant immunoreactive protein is unaltered regardless of the cDNA expressed. The cleaved ACBP/SREBP-2 migrated at –15 kDa.

Fig. 2. Immunoblot analysis of 293 cells transfected with truncated forms of epitope tagged ACBP/SREBP-2 fusion proteins. A, schematic diagram depicting the location of stop mutations in the HSV-tagged ACBP/SREBP-2 protein. Numbers refer to the amino acid residue in wild-type human SREBP-2. Residue 473 denotes the first amino acid of wild-type human SREBP-2 in the fusion protein. B, immunoblot analysis. Cells were transfected with 4 μg/dish of wild-type or the indicated mutant pTK-HSV-ACBP/BP2 plasmid and incubated for 24 h in medium B in the absence of sterols as described under “Experimental Procedures.” Aliquots of the cytosol (100 μg of protein for lanes 1–3, 5, 7, and 9–11 and 1–5 μg for lanes 4, 6, and 8) were adjusted to 100 μg of total protein by the addition of mock-transfected cytosol. Protein samples were subjected to Tricine SDS-PAGE and immunoblot analysis with 0.5 μg/ml IgG-HSV-Tag as described under “Experimental Procedures.” The filter was exposed to film for 20 s. The migration of a 56-kDa irrelevant immunoreactive protein is unaltered regardless of the cDNA expressed. The cleaved ACBP/SREBP-2 migrated at –15 kDa.

To determine the amino acid residues that are required for cleavage of SREBP-2 at Site-2, we made a systematic series of mutations in which an alanine or phenylalanine residue was substituted individually for most of the amino acids in the first transmembrane segment that are conserved in the seven mammalian SREBPs (Fig. 3). 293 cells were transfected with a cDNA encoding wild-type or mutant human SREBP-2 that was tagged at its NH$_2$ terminus with the HSV tag, and the cells were incubated in the absence or presence of sterols. Nuclear extracts and membrane fractions from the transfected cells were subjected to SDS-PAGE and immunoblotted with an antibody against the NH$_2$-terminal HSV epitope tag. The results from analysis of all 15 mutations are summarized in Fig. 3. The data reveal that all mutants were cleaved in a fashion similar to that of wild-type human SREBP-2.

During the course of these studies, we noticed that ACBP lacks cysteine residues and that the first cysteine in the fusion protein occurs at amino acid 485 of SREBP-2, which is within the range previously defined as the site of cleavage (see Fig. 2). Moreover, the data of Fig. 3 indicated that this cysteine and the other residues near the presumed cleavage site could be replaced with alanine or phenylalanine without affecting cleavage. We hypothesized that replacement with cysteine would be equally well tolerated. To take advantage of this situation, we first prepared a series of plasmids encoding ACBP/SREBP-2 fusion proteins with an NH$_2$-terminal HSV-epitope tag followed by 8 histidines and then performed the procedure illus-
Amino acid alignment of the first transmembrane sequence of human (19), hamster (25, 26), rat (27), mouse (H. Shimano, J. D. Horton, M. S. Brown, and J. L. Goldstein, unpublished data), and *D. melanogaster* (28) SREBP's is shown at the top. For reference, the first amino acid in the first transmembrane sequence of human SREBP-2 is 482 and the last amino acid is 502. Highlighted letters denote amino acid residues that are identical in all eight SREBPs. Boxed letters denote amino acid residues that are identical in the seven mammalian sequences but not in the *Drosophila* sequence. Residues individually mutated to alanine (A) or phenylalanine (F) in human SREBP-2 are indicated below the alignment. The cleavage of each mutant SREBP-2 relative to that of wild-type human SREBP-2, as determined by immunoblot analysis of transfected 293 cells, is shown at the right. All of the mutants are given a value of 4+, indicating that cleavage of these proteins was equivalent to that of wild-type human SREBP-2.

As shown in Fig. 4, each fusion protein had a cysteine residue that was located either before or after the suspected site of cleavage. After partial purification by Ni-NTA agarose chromatography, the cytosolic NH2-terminal fragment was reacted with MPB, which attaches biotin to cysteine sulfhydrys. Excess MPB was removed by a second round of Ni-NTA chromatography, and the MPB-derivatized proteins were trapped by binding to streptavidin-agarose. The supernatant and pellet fractions from the streptavidin-agarose precipitation were subjected to SDS-PAGE and immunoblotted with an antibody directed against the HSV epitope tag. If the cysteine is on the NH2-terminal side of Site-2, the NH2-terminal fragment should be found in the streptavidin pellet. If the cysteine is distal to Site-2, the NH2-terminal fragment should not be derivatized with biotin and should be found in the streptavidin supernatant.

As shown in Fig. 5, residues 483–486 of the ACBP/SREBP-2 fusion protein were individually replaced with cysteine. To remove any ambiguity, we replaced cysteine 485 with alanine (A) or phenylalanine (F) in human SREBP-2 and determined the cleavage of each mutant SREBP-2 relative to that of wild-type human SREBP-2, as determined by immunoblot analysis of transfected 293 cells, is shown at the right. All of the mutants are given a value of 4+, indicating that cleavage of these proteins was equivalent to that of wild-type human SREBP-2.
proteins with cysteines at each of the positions between residues 483 and 486 (Fig. 7A), and the cytosolic NH₂-terminal fragments were subjected to the procedure of Fig. 4. As shown in Fig. 7B, when the first cysteine was at position 491, the fusion protein appeared in the supernatant after streptavidin precipitation (lanes 3 and 4), confirming that cysteine 491 is distal to Site-2. When the first cysteine was at position 483 or 484, the protein was found in the pellet after streptavidin precipitation (Fig. 7B, lanes 5–8). When the first cysteine was at position 485 or 486, the protein was in the supernatant (Fig. 7B, lanes 9–12). These data indicate that Site-2 proteolysis occurs at the peptide bond between residues 484 and 485 (Fig. 7A).

We considered the possibility that cysteine at the extreme COOH-terminus of the NH₂-terminal fragment might be resistant to MPB derivatization. To rule out this possibility, we produced a truncated ACBP/SREBP-2 that terminates immediately following cysteine 485 (Fig. 7A, Stop-486 cDNA). Following derivatization with MPB and streptavidin precipitation, this protein was found in the pellet (Fig. 7B, lanes 13 and 14), indicating that a cysteine at the COOH-terminal position of ACBP/SREBP-2 can be modified with MPB.

We quantified the chemiluminescence signal of ACBP/SREBP-2 protein in the supernatant or pellet fraction for each of the mutations (Fig. 7C). The results yielded a clear discrimination. In each case over 90% of the protein was found in either the pellet or the supernatant. These data indicate that S2P cuts the intermediate SREBP-2 clearly at a single peptide bond and that there is no significant further shortening of the protein.

Intramembrane Cleavage of SREBP

The next experiment was designed to determine whether Site-2 proteolysis of full-length human SREBP-2 also occurs at the Leu⁴⁸⁴-Cys⁴⁸⁵ peptide bond. For this purpose, we took advantage of the observation that there is only a single cysteine in the NH₂-terminal domain of SREBP-2 (Ref. 19). We mutated this cysteine, at residue 51, to alanine. We then made cDNAs encoding SREBP-2 (C51A) with the first cysteine at position 484 or 485. Immunoblotting of nuclear extracts confirmed that cysteine 491 is distal to Site-2 proteolysis of full-length human SREBP-2 also occurs at the Leu⁴⁸⁴-Cys⁴⁸⁵ peptide bond. For this purpose, we took advantage of the observation that there is only a single cysteine in the NH₂-terminal domain of SREBP-2 (Ref. 19). We mutated this cysteine, at residue 51, to alanine. We then made cDNAs encoding SREBP-2 (C51A) with the first cysteine at position 484 or 485. Immunoblotting of nuclear extracts confirmed that cysteine 491 is distal to Site-2 proteolysis of full-length human SREBP-2 also occurs at the Leu⁴⁸⁴-Cys⁴⁸⁵ peptide bond. For this purpose, we took advantage of the observation that there is only a single cysteine in the NH₂-terminal domain of SREBP-2 (Ref. 19). We mutated this cysteine, at residue 51, to alanine. We then made cDNAs encoding SREBP-2 (C51A) with the first cysteine at position 484 or 485. Immunoblotting of nuclear extracts confirmed that cysteine 491 is distal to Site-2 proteolysis of full-length human SREBP-2 also occurs at the Leu⁴⁸⁴-Cys⁴⁸⁵ peptide bond.
sequence immediately preceding the first transmembrane segment. Previously, we found that replacement of these four residues with AS abolished cleavage at Site-2 (Ref. 12). As a starting point for the further definition of the sequence responsible for recognition of Site-2, we mutated D\(^{478}\)RSR to AAAA, preserving the length of the segment but eliminating the four polar residues. In the experiment of Fig. 9, wild-type human SREBP-2 was processed to produce the nuclear NH\(_2\)-terminal fragment in a sterol-regulated manner (Fig. 9B, lanes 3 and 4, designated M), but the D\(^{478}\)RSR → AS and the D\(^{478}\)RSR → AAAA mutations failed to undergo Site-2 proteolysis, and therefore they generated the membrane-bound intermediate forms (Fig. 9B, lanes 5–8, designated I). When only the initial aspartic acid was preserved (DAAA), Site-2 cleavage was also abolished (Fig. 9B, lanes 9 and 10). These observations suggest that one or both of the arginines in the DRSR sequence is essential for Site-2 cleavage.

To study each of the arginines in the DRSR sequence separately, we prepared plasmids encoding mutant forms of SREBP-2 in which the first or second arginine was replaced with alanine (DASR and DRSA, respectively). Both of these proteins were cleaved by the Site-2 protease to generate mature nuclear forms (Fig. 9B, lanes 11 and 13), indicating that either arginine is sufficient. To determine whether the position of the arginine affects the precise site of cleavage, we prepared versions of the DASR and DRSA plasmids in which cysteine 51 was replaced with alanine. Cells were harvested and processed in the legend to Fig. 6 and under “Experimental Procedures.” Cells were harvested, fractionated, and the cleaved NH\(_2\)-terminal domains were purified by Ni-NTA chromatography, treated with MPB, and precipitated with streptavidin-agarose as described in the legend to Fig. 6 and under “Experimental Procedures.”

The immunoblot was quantified as described under “Experimental Procedures.” Equal aliquots (50% of total volume) of the supernatant (S) and pellet (P) were subjected to SDS-PAGE and immunoblot analysis with 0.5 μg/ml IgG-HSV-Tag. The filters were exposed to film for 45 s. ACBP/SREBP-2 proteins with a cysteine NH\(_2\)-terminal to cleavage at Site-2 are derivatized with MPB and found in the supernatant (S). The other bands are present in mock-derivatized ACBP/SREBP-2 protein bound to streptavidin. The immunoblot was quantified as described under “Experimental Procedures.” Each bar represents the percentage of total ACBP/SREBP-2 found in the supernatant (closed bars) or pellet (hatched bars). The results shown are representative of three independent transfection experiments.

![Figure 7](image-url)

**Fig. 7.** MPB derivatization of cysteine residues in ACBP/SREBP-2 fusion proteins: localization of Site-2 cleavage within the first transmembrane sequence. A, schematic diagram of the alanine-modified HSV, 8His-tagged ACBP/SREBP-2 fusion protein. Residue 473 denotes the first amino acid residue in wild-type SREBP-2. All fusion proteins, except that numbered 485, contain alanine substituted for cysteine at residue 485. cDNAs encoding proteins with the indicated mutation are shown below the sequence. The position of the first cysteine in each fusion protein is shown. B, immunoblot analysis of ACBP/SREBP-2 treated with MPB. 293 cells were co-transfected with the indicated version of pTK-HSV-8His-BP2 and three other plasmids as described in the legend to Fig. 6 and under “Experimental Procedures.” Cells were harvested and fractionated, and the cleaved NH\(_2\)-terminal domains were purified by Ni-NTA chromatography, treated with MPB, and precipitated with streptavidin-agarose as described under “Experimental Procedures.” Equal aliquots (50% of total volume) of the supernatant (S) and pellet (P) were subjected to SDS-PAGE and immunoblot analysis with 0.5 μg/ml IgG-HSV-Tag. The immunoblot was quantified as described under “Experimental Procedures.” Equal aliquots (50% of total volume) of the supernatant (S) and pellet (P) were subjected to SDS-PAGE and immunoblot analysis with 0.5 μg/ml IgG-HSV-Tag antibody. The filters were exposed to film for 15 s. M and P denote the cleaved NH\(_2\)-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. C, quantification of the amount of MPB-derivatized SREBP-2 bound to streptavidin. The immunoblot from B was quantified as described under “Experimental Procedures.” Each bar represents the percentage of total SREBP-2 found in the supernatant (closed bars) or pellet (hatched bars). The results shown are representative of three independent transfection experiments.

![Figure 8](image-url)

**Fig. 8.** MPB derivatization of cysteine residues in SREBP-2: localization of Site-2 cleavage to the leucine 484-cysteine 485 bond. A, immunoblot analysis of epitope-tagged SREBP-2. 293 cells were transfected with 4 μg/dish of either wild-type pTK-HSV-8His-BP2 (denoted as Cys\(^{485}\)) or the indicated mutant plasmid (Cys\(^{484}\) or Cys\(^{485}\)) in which cysteine 51 has been replaced with alanine. Cells were incubated in the absence (−) or presence (+) of 1 μg/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol (sterols) as indicated. Cells were harvested and fractionated as described under “Experimental Procedures.” Aliquots of the nuclear extract (60 μg of protein) and membranes (80 μg of protein) were subjected to SDS-PAGE and immunoblot analysis with 0.5 μg/ml IgG-HSV-Tag antibody. The filters were exposed to film for 15 s. M and P denote the cleaved NH\(_2\)-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. B, immunoblot analysis of SREBP-2 derivatized with MPB. 293 cells were co-transfected with the indicated version of pTK-HSV-8His-BP2 together with three other plasmids as described in the legend to Fig. 6 and under “Experimental Procedures.” Cells were harvested and processed as described in the legend to Fig. 7 except that the filter was exposed to film for 60 s. C, quantification of the amount of MPB-derivatized SREBP-2 bound to streptavidin. The immunoblot from B was quantified as described under “Experimental Procedures.” Each bar represents the percentage of total SREBP-2 found in the supernatant (closed bars) or pellet (hatched bars). The results shown are representative of three independent transfection experiments.
The cysteine panning technique that we developed for these studies has revealed that Site-2 cleavage of human SREBP-2 occurs at the Leu484-Cys485 bond, which is located three residues to the COOH-terminal side of the DRSR sequence that is required for cleavage. This Leu-Cys sequence is conserved not only in all seven mammalian SREBPs, but also in SREBP from Drosophila melanogaster (Fig. 3; Refs. 19 and 25–28).

The Leu-Cys cleavage site is within the range that was defined previously on the basis of the size of the mature NH₂-terminal fragment (12). This site is also just within the sequence that was previously defined as the first membrane-spanning segment on the basis of hydrophobicity plots (9). These plots predict that the DRSR sequence marks the end of the NH₂-terminal cytoplasmic domain (see Fig. 3). In the mammalian SREBP-2s, the DRSR is followed by the hydrophobic sequence ILLC (LALC in SREBP-1s), which initiates a long hydrophobic stretch that is predicted to constitute the first transmembrane domain. We believe that the transmembrane domain terminates at the conserved tryptophan that is 22 residues downstream from the DRSR sequence in all the SREBPs except human SREBP-1 and Drosophila SREBP where this position is occupied by arginine (Fig. 3). The tryptophan or arginine is invariably followed by glycine. An additional glycine or proline is frequently found in the immediate downstream sequence. Both of these residues would be expected to disrupt the transmembrane a-helix. In the first transmembrane protein to be studied by crystallography, the photosynthetic reaction center of Rhodopseudomonas viridis, two-thirds of the tryptophan residues of the L and M subunits were located at the junction between the hydrophobic surface and the polar transition zone or in the polar regions near the hydrophobic surface (29).

We emphasize that there is no direct evidence that the Leu484-Cys485 bond actually lies within the phospholipid bilayer. We cannot exclude the possibility that this bond lies on the surface of the membrane and not within it.

If the Leu484-Cys485 peptide bond does lie within the membrane, it would be just beneath the polar head groups of the phospholipids. The bond might be close enough to the bilayer surface so that the Site-2 protease could surround it, sequencing it from the phospholipids and permitting attack by a molecule of water. Such an intramembrane location would be consistent with the predicted structure of the protein that has been postulated to be the Site-2 protease. This is a highly unusual polytopic membrane protein with a consensus metalloprotease zinc-binding site (HEIGH) that has a unique location within an otherwise hydrophobic region of the sequence (15). This zinc atom might be in an ideal position to cleave a peptide bond that was located just beneath the surface of the lipid bilayer.

**DISCUSSION**

**Fig. 9. Sequence requirement of DRSR region in Site-2 cleavage of human SREBP-2.** A, schematic diagram of the wild-type sequence of epitope-tagged SREBP-2, showing Site-2 cleavage between residues 484 and 485 of the first transmembrane region (boxed amino acids). cDNAs (designated A-G) encoding proteins with the indicated mutations are shown below the sequence. Mutated cDNAs either replace or modify the DRSR region of SREBP-2. For the MPB derivatization reaction, the wild-type cysteine 485 is either left intact (cDNAs E and G) or mutated to an alanine with a cysteine substituted at residue 484 (cDNAs D and F). B, immunoblot analysis of membrane fractions and nuclear extracts from 293 cells transfected with 4 μg/dish of the indicated version of SREBP-2 as described under “Experimental Procedures.” Cells were incubated in the absence (—) or presence (+) of 1 μg/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol (sterols) as indicated. Cells were harvested and processed as described in the legend to Fig. 8 except that the filter with membrane fractions was exposed to film for 10 s and the filter with nuclear extracts was exposed for 15 s. C, lanes 1–4, immunoblot analysis of membrane fractions from 293 cells transfected with 4 μg/dish of the indicated version of SREBP-2 derivatized with MPB. 293 cells were co-transfected along with three other plasmids as described in Fig. 6 and under “Experimental Procedures.” Both the wild-type and mutant versions of pTK-HSV-SHisBP2 plasmids encode proteins in which cysteine 51 is replaced with alanine, removing the only cysteine in the NH₂-terminal domain of SREBP-2. Cells were harvested and processed as described in Fig. 7 except that the filter was exposed to film for 15 s. Wild-type and mutant versions of SREBP-2 contain cysteines in position 484 or 485 as indicated. D, quantification of the amount of MPB-derivatized SREBP-2 bound to streptavidin. The immunoblot from Fig. 7 except that the filter was exposed to film for 15 s. Wild-type and mutant versions of SREBP-2 contain cysteines in position 484 or 485 as indicated. D, quantification of the amount of MPB-derivatized SREBP-2 bound to streptavidin. The immunoblot from Fig. 7 except that the filter was exposed to film for 15 s.
A major unresolved question relates to the specificity of the recognition site that directs the Site-2 protease to the SREBP. The current data suggest that only a single juxtamembranous arginine is required. It is possible that even this residue is not directly recognized by the Site-2 protease, but that its role is to position the transmembrane a-helix. Consistent with this notion are the observations of Fig. 9, which demonstrate that the cleavage site remains at the Leu<sup>484</sup>-Cys<sup>485</sup> bond whether the arginine is not part of a peptide recognition site. The postulated transmembrane sequences of all seven mammalian SREBPs shown in Fig. 3 contain 10 identically conserved residues, six of which are leucines that are spaced an average of three residues apart (LXXLXXLXXXLXXL). Remarkably, most of these conserved leucines could be replaced with alanine without affecting Site-2 cleavage of human SREBP-2 (Fig. 3). The same was true for the other four conserved residues in the transmembrane segment, including the phenylalanine and the proline that are conserved in the Drosophila sequence as well as the mammalian sequences (Fig. 3). Even though the Leu<sup>484</sup>-Cys<sup>485</sup> is conserved as far back as Drosophila, these residues are not required for cleavage. Both residues could be replaced with phenylalanine without altering the cleavage (mutant 15 in Fig. 3). Additional studies will be required to determine how the Site-2 protease discriminates SREBP-2 from the many other transmembrane proteins that have arginine residues at the water-lipid interface.

We note that the relative position of Site-2 in SREBP differs from the relative position of the cleavage site in the amyloid precursor protein, the only protein in which intramembrane cleavage previously has been documented (30). Amyloid precursor protein is cleaved predominantly at two sites that are two residues apart, giving rise to peptides of 40 and 42 amino acids in length, respectively. Both sites are near the middle of the postulated transmembrane sequence, which contrasts with the junctional location of the SREBP Site-2. The enzyme that cleaves amyloid precursor protein within the membrane, termed the y-secretase, has not yet been isolated. At present it is unclear as to whether a single y-secretase makes both cleavages, or whether two y-secretases exist. The double site of cleavage of amyloid precursor protein differs from the single intramembrane site of cleavage that is documented by the cysteine panning technique used in these studies. Indeed, one advantage of this technique is that it gives a quantitative readout of the percentage of observed cleavage that occurs at the Leu<sup>484</sup>-Cys<sup>485</sup> bond. These data indicate that this site of cleavage represents more than 90% of the cleavage that occurs within SREBP-2 and that there is no trimming back of the cleaved peptide beyond residue 484. Inasmuch as this technique measures only the final cleavage site, we cannot exclude the unlikely possibility that intramembrane cleavage of SREBP-2 is initiated at a site that is distal to the 484/485 bond and that there is further precise trimming of all cleaved molecules back to residue 484.

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