Cleavage of Sterol Regulatory Element-binding Proteins (SREBPs) at Site-1 Requires Interaction with SREBP Cleavage-activating Protein

EVIDENCE FROM IN VIVO COMPARISON STUDIES*

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Sterol regulatory element-binding proteins (SREBPs) are membrane-bound transcription factors that promote lipid synthesis in animal cells. They are embedded in the membranes of the endoplasmic reticulum (ER) in a helical hairpin orientation and are released from the ER by a two-step proteolytic process. Proteolysis begins when the SREBPs are cleaved at Site-1, which is located at a leucine residue in the middle of the hydrophobic loop in the lumen of the ER. Sterols suppress Site-1 cleavage, apparently by interacting with a polytopic membrane protein designated SREBP cleavage-activating protein (SCAP). SREBPs and SCAP are joined together in ER membranes through interaction of their cytoplasmic COOH-terminal domains. Here we use an in vivo competition assay in transfected cells to show that the SREBP-SCAP complex is essential for Site-1 cleavage. Overexpression of the truncated COOH-terminal domains of either SREBP-2 or SCAP disrupted the complex between full-length SREBP-2 and SCAP as measured by co-immunoprecipitation. This resulted in a complete inhibition of Site-1 cleavage that was restored by concomitant overexpression of full-length SCAP. The transfected COOH-terminal domains also inhibited the transcription of a reporter gene driven by an SRE-containing promoter, and this, too, was restored by overexpression of full-length SCAP. We interpret these data to indicate that the SREBP-SCAP complex directs the Site-1 protease to its target in the lumenal domain of SREBP and that disruption of this complex inactivates the Site-1 cleavage reaction.

* This work was supported by Research Grant HL20948 from the National Institutes of Health and by the Perot Family Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: SREBP, sterol regulatory element-binding protein; bp, base pair(s); CHO, Chinese hamster ovary; CMV, cytomegalovirus; ER, endoplasmic reticulum; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HSV, herpes simplex virus; kb, kilobase(s); LDL, low density lipoprotein; PC, polymerase chain reaction; PSS, prolactin signal sequence; SCAP, SREBP cleavage-activating protein; SRE-1, sterol regulatory element-1; TK, thymidine kinase.

NH₂-terminal domains of the SREBPs, which travel to the nucleus where they activate transcription of genes encoding the low density lipoprotein (LDL) receptor; multiple enzymes of the cholesterol biosynthetic pathway, including 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase, HMG-CoA reductase, farnesyl diphosphate synthase, and squalene synthase; and enzymes of fatty acid biosynthesis, including acetyl-CoA carboxylase and fatty acid synthase. When sterols accumulate in cells, the proteolytic processing of SREBPs is inhibited, the NH₂-terminal domains remain bound to membranes, and transcription of the target genes declines.

Recent experiments have begun to elucidate the details of the two-step proteolytic process and the mechanism for its regulation by sterols (1–5). A key aspect is the three-domain structure of the SREBP precursors (1). The NH₂-terminal domains of the SREBPs are ~480 amino acids in length and contain a classic basic helix-loop-helix-leucine zipper motif and an acidic transcription activation domain, similar to the ones that are found in numerous transcription factors. The NH₂-terminal domain is followed by a membrane anchor domain consisting of two hydrophobic sequences, each of which spans the ER membrane and is separated from the other by a short hydrophilic loop of ~30 residues. The membrane anchor domain is followed by a long COOH-terminal extension of ~590 amino acids, which is designated as the regulatory domain. The SREBP precursors are oriented so that the NH₂-terminal and COOH-terminal domains face the cytoplasm, and only the short hydrophilic loop projects into the ER lumen (5).

In sterol-deprived cells, the proteolytic process is initiated by an enzyme that cleaves the SREBP precursors at Site-1, which is located in the middle of the hydrophilic lumenal loop (1–3). This cleavage separates the NH₂-terminal and COOH-terminal domains, but each remains membrane-bound, owing to its transmembrane sequence. At this point a second protease cleaves the NH₂-terminal fragment at Site-2 within its membrane-spanning region, liberating the NH₂-terminal domain so that it can enter the nucleus (1, 2). The Site-1 cleavage enzyme is directly regulated by sterols; it acts only in sterol-depleted cells, and it is inhibited by sterols. The Site-2 enzyme is not controlled directly by sterols, but it can act only after cleavage at Site-1, and its action is therefore restricted effectively to sterol-depleted cells (1, 2).

The Site-1 enzyme recognizes the sequence RXXL, which is conserved in all known mammalian and Drosophila SREBPs. The enzyme cleaves after the leucine of this sequence (1, 3). In human SREBP-2, cleavage is abolished when the arginine of the RSVL sequence is changed to alanine. Site-1 cleavage also requires the COOH-terminal domain of SREBPs. When this domain is deleted, the Site-1 enzyme will no longer cleave SREBP-2 even though the RSVL sequence is still present (4).
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For this reason, we refer to the COOH-terminal domain of the SREBPs as the regulatory domain.

The activity of the Site-1 cleavage enzyme is proposed to be controlled by a membrane-bound protein called SREBP cleavage-activating protein (SCAP) (6). SCAP consists of two domains: 1) an NH2-terminal membrane anchor of \( \sim 730 \) amino acids composed of eight putative membrane-spanning segments; and 2) a hydrophilic COOH-terminal segment of \( \sim 546 \) amino acids that contains at least four "WD repeats." These repeats, each about 40 residues in length, are found in many proteins that engage in protein-protein interactions (7). Like the SREBPs, SCAP is bound to membranes of the ER and nuclear envelope (4).

Co-immunoprecipitation assays show that the WD repeat domain of SCAP is bound to the COOH-terminal regulatory domains of the SREBPs (4). Genetic evidence indicates that SCAP is responsible for the sterol regulation of Site-1 cleavage. Thus, a dominantly acting point mutation in SCAP, D443N, was identified as the cause of sterol resistance in several independently derived lines of mutant Chinese hamster ovary (CHO) cells in which Site-1 cleavage was no longer repressed by sterols (6, 8). Transfection of a cDNA encoding the D443N mutant version of SCAP into wild-type CHO cells reproduced the inhibitory effects of sterols, and hence the cleavage at Site-1 can no longer be down-regulated.

The notion that SCAP is part of the sterol-sensing mechanism is supported by the finding that the NH2-terminal membrane attachment domain of SCAP bears significant sequence resemblance to the membrane attachment domain of HMG-CoA reductase (6). The latter domain serves as a sterol sensor, allowing HMG-CoA reductase to be degraded rapidly when the sterol content of the ER rises (9, 10). A sequence resembling the membrane attachment domain of HMG-CoA reductase is present in many proteins that are postulated to interact with sterols (SCAP, HMG-CoA reductase, Niemann-Pick type C1 protein, and the two fragments were ligated to generate pCMV-PSS/BP2-(504–1141).

We obtained monoclonal antibody HSV-Tag™ (IgG1) from Novagen, monoclonal anti-FLAG M2 (IgG4) from Eastman Kodak Co., and a polyclonal affinity-purified donkey anti-mouse IgG from Jackson Immunoresearch Laboratories. IgG-106, a mouse monoclonal antibody directed against the COOH terminus of human SREBP-2 (amino acids 853–1141; Ref. 5); IgG-ID5, a mouse monoclonal antibody against hamster SCAP (amino acids 540–707; Ref. 4); and IgG-R139, a rabbit polyclonal antibody against hamster SCAP (amino acids 54–277 and 540–707 and Ref. 4), were prepared as described in the indicated reference. Luciferase and \( \beta \)-galactosidase assay kits were obtained from Promega and Stratagene, respectively. Other reagents were obtained from sources as described previously (3–5, 13).

Construction of Plasmids—All expression vectors were driven by the cytomegalovirus (CMV) promoter-enhancer contained in the pcDNA3 vector (Invitrogen). The structures of all plasmid constructs described below were confirmed by sequencing all ligation joints.

The expression vector pCMV-PSS/BP2-(504–1141) encodes a fusion protein consisting of a modified region of the bovine prolactin signal sequence (amino acids 504–533) and the two fragments were ligated to generate pCMV-PSS/BP2-(504–1141). The prolactin signal sequence was modified by the substitution of an asparagine residue at \( 1 \) in place of serine, as denoted by the underline. pCMV-PSS/BP2-(504–1141) was constructed as follows. First, a 60-bp fragment encoding the prolactin signal sequence (PSS) followed by the FLAG epitope tag was isolated by KpnI and SalI digestion of a plasmid construct in pBluescript encoding a PSS/FLAG/human thrombin receptor fusion protein (Ref. 14; provided by Dr. Shaun R. Coughlin, University of California, San Francisco). The sequence corresponding to amino acids 504–533 of human SREBP-2 (15) was amplified by PCR of pTK-HSV-BP2 (13) with a pair of primers, 5′-GGGCTCTGTCTCTCCTGTTTGCTTCTCCTTTCACTCTGGAAACAG-GGTCGACGGAGGGGCCCACGACTCTGACCA-3′ encoding amino acids 504–533 preceded by an EcoRI site. The PCR fragment was digested with SalI and EcoRI, and the 90-bp fragment was isolated. The above two fragments were cloned into the KpnI-EcoRI sites of pCMV-PSS/BP2-(504–1141).

The expression vector pCMV-PSS/BP2-(504–1141) encodes a fusion protein consisting of amino acids 1–29 of cytochrome P450 2C1, two novel amino acids (ID). pCMV-PSS/BP2-(504–1141) was constructed as follows. First, a 60-bp fragment encoding the prolactin signal sequence (PSS) followed by the FLAG epitope tag was isolated by KpnI and SalI digestion of a plasmid construct in pBluescript encoding a PSS/FLAG/human thrombin receptor fusion protein (Ref. 14; provided by Dr. Shaun R. Coughlin, University of California, San Francisco). The sequence corresponding to amino acids 504–533 of human SREBP-2 (15) was amplified by PCR of pTK-HSV-BP2 (13) with a pair of primers, 5′-GGGCTCTGTCTCTCCTGTTTGCTTCTCCTTTCACTCTGGAAACAG-GGTCGACGGAGGGGCCCACGACTCTGACCA-3′ encoding amino acids 504–533 preceded by an EcoRI site. The PCR fragment was digested with SalI and EcoRI, and the 90-bp fragment was isolated. The above two fragments were cloned into the KpnI-EcoRI sites of pCMV-PSS/BP2-(504–1141).

The expression vector pCMV-PSS/BP2-(504–1141)SSS/NNT encodes a FLAG epitope-tagged prolactin signal sequence/SREBP-2 fusion protein in which serine 515 in the loop region of SREBP-2 was replaced by site-directed mutagenesis (13, 16), with a novel amino acid sequence containing two glycosylation sites, NSSGSSNGN. Other derivatives of pCMV-PSS/BP2 were also constructed by site-directed mutagenesis (13, 16).

The expression vector pCMV-PSS/BP2-(504–1141)SSS/NNT encodes a fusion protein consisting of amino acids 1–29 of cytochrome P450 2C1 (17), two novel amino acids (ID) encoded by a BapDI restriction site, and amino acids 555–1141 of human SREBP-2. pCMV-PSS/BP2-(505–1141) was constructed as follows. First, a complementary oligonucleotides (top strand) 5′-CTAGGTTATGATGATCCTGTTGTTTGCTGTTTG-GGCTGCTTGGCCTTGGGCTTGGCCTTGGGCTTGGAAGCTGAGCCTATCAGGAGGGAAGGAGGAAAGCTTATGGGGGAGGGAAGCTTAT-3′, which contained 5′ NheI and 3′ BapDI cohesive ends, were annealed as described previously (5). This oligonucleotide encodes the sequence for amino acids 1–29 of cytochrome P450 2C1 (MDPVVVVLGLCLSCLLLLLWKKQSYGGKKL) and two novel amino acids (ID). pCMV-HSV-BP2-(505–1141) (4) was digested with NheI and BapDI, and the 90-bp fragment encoding two tandem copies of the HSV epitope was replaced with the amino acids 1–29 of the P450 2C1 sequence.

pCMV-P450-TM/SCAP-(731–1276) encodes a fusion protein consisting of amino acids 1–29 of cytochrome P450 2C1, two novel amino acids (RT) encoded by a BsiWI restriction site, and amino acids 731–1276 of hamster SCAP (6). To generate pCMV-P450-TM/SCAP-(731–1276), a
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PCR fragment was amplified from pC1-GAL (Ref. 17; kindly provided by Byron Kemper, University of Illinois at Urbana-Champaign), a CMV-driven expression vector encoding a hybrid protein in which amino acids 1–29 of P450 2C1 are fused to the NH2 terminus of Escherichia coli BsiWI restriction site. (In this paper, pC1-GAL is referred to as pCMV-P450-TM/Gal.) PCR amplification was carried out with the following primers: 5'-CCCATGTCGGAGCTCAACAA-3' (annealing to a sequence in the CMV promoter) and 5'-ATGCTATACAGATTTCTCCCTCCATATCCTGTGTTTC-3' (containing codons 20–29 of cytochrome P450 2C1 preceded by a BsiWI restriction site). The PCR fragment was cut with Ndel and BsiWI and used to replace the Ndel-BsiWI fragment of pDNA2-CMV-SCAP (NAS176), a CMV-driven expression vector encoding human SREBP-2 (13), and a SacI-EcoRI intermediate plasmid containing the coding sequence of amino acids 1–7 of human cytochrome B5 reductase (18), followed by a BsiWI restriction site and codons 731–1276 of SCAP.

Other Plasmids—pTK-HSV-BP2, a herpes simplex virus thymidine kinase-driven expression vector encoding human SREBP-2 (13), and pCMV-SCAP, a CMV-driven expression vector encoding hamster SCAP (4), were prepared as described in the indicated reference. pCMVβ-gal, a plasmid encoding a CMV promoter-driven β-galactosidase reference gene, was obtained from Stratagene, pSRE-Luc, a luciferase reporter plasmid driven by a promoter consisting of three tandem copies of repeats 2–3 of the LDL receptor promoter (SRE-1) plus the adenovirus E1B TATA box was constructed as described previously (6).

Detection of SREBP Cleavage—

Cells—Monolayers of M19 cells are a mutant line of CHO-K1 cells (19, 20). The cells were grown in monolayer culture as described previously (5). Three h after transfection, the cells were switched to medium B (medium A containing 10% newborn calf lipoprotein-deficient serum, 100 units/ml penicillin, and 0.5% SDS and 1% (v/v) sodium dodecyl sulfate) and fed with 2 ml of medium B in the absence or presence of sterols as described above. After 20 h, the cells in each well were lysed with 0.2 ml of 1% Triton X-100 and 100 µg/ml streptomyacin sulfate supplemented with 10% (v/v) fetal calf serum. On day 2, the cells were transfected with the indicated plasmids using the MBs kit (Stratagene) method as described previously (5). 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 or presence of sterols (1 µM/µl 25-hydroxycholesterol plus 10 µM/µl cholesterol added in a final concentration of 0.2% ethanol) as indicated in the legends. After incubation for 20 h, the cells received cholesterol added in a final concentration of 0.2% ethanol) as indicated in the legends. After incubation for 20 h, the cells were harvested, and the pooled cells from two dishes were incubated directly with neuraminidase or boiled for 5 min in the presence of 0.5% SDS and 1% (v/v) β-mercaptoethanol for 5 min. The pooled cell suspension from two dishes was allowed to swell in hypotonic buffer A (10 mM Hepes–KOH at pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and a mixture of protease inhibitors; Ref. 5) for 30 min at 0 °C, passed through a 22.5-gauge needle 30 times, and the pooled cell suspension from two dishes was centrifuged at 105,000 g for 30 min at 4 °C in a Beckman TLA 105.3 rotor. The 105,000 g pellet was washed once with buffer A and resuspended in 180 µl of buffer B (medium A containing 1% (v/v) Triton X-100 without protease inhibitors). Cleavage of SREBPs Requires SREBP-2 to compete for the Site-1 cleavage reaction, we prepared a series of expression vectors encoding fusion proteins in which the NH2-terminal domain and the first transmembrane segment of SREBP-2 were replaced by PSS followed by a FLAG epitope tag (Fig. 1). This was joined to SREBP-2 at residue 504, which marks the beginning of the hydrophilic luminal loop. In the construct shown in Fig. 1, the SREBP-2 sequence extended to the normal COOH terminus of the protein (residue 1141), thereby including the COOH-terminal regulatory domain. To prevent cleavage of the PSS by signal peptidase, we changed the serine adjacent to the signal sequence cleavage site to aspartagine, which is known to abolish cleavage by signal peptidase (21). The cDNA encoding the fusion protein was transfected into human embryonic kidney 293 cells. The cells were incubated in a sterol-containing medium to repress cleavage at Site-1. After 23 h, cell membranes were isolated, and the proteins were subjected to SDS-PAGE and immunoblotted with an antibody against the COOH-terminal regulatory domain of SREBP-2. The PSS/BP2 fusion protein was visualized as a single band of 68 kDa, which corresponded to the expected mass of the fusion protein at Site-1, which is the size predicted if the fusion protein had been cleaved at Site-1 (lane 1). To trigger cleavage of the fusion protein at Site-1, we cotransfected a cDNA encoding wild-type SCAP driven by the strong CMV promoter. High expression of wild-type SCAP is known to relieve sterol suppression of Site-1 cleavage (4, 6). Under these conditions we observed a new band with an apparent molecular mass of 62 kDa, which is the size predicted if the fusion protein had been cleaved at Site-1, i.e., at L522 in SREBP-2 (lane 2). This apparent cleavage product was not seen when PSS/BP2 contained an alanine in place of arginine at position 519, a substitution that is known to abolish cleavage at Site-1 (lane 3). To confirm that the PSS/BP2 fusion protein was positioned properly at the ER membrane, we injected a DNA sequence encoding a short peptide that contains two consensus sites for N-linked glycosylation (designated NSS/NGT in Fig. 2). The sequence was inserted into the luminal loop on the NH2-terminal side of Site-1. This protein appeared as a band at 72 kDa when the membrane fraction of transfected cells was blotted with an antibody against the SREBP-2 COOH-terminal do-
Fig. 1. Cleavage of prolactin signal sequence/SREBP-2 fusion protein at Site-1 in transfected 293 cells. The diagram shows the fusion protein encoded by pCMV-PSS/BP2-(504–1141). The serine residue at +1 of the prolactin signal sequence was changed to asparagine (denoted by the bold letter N) to prevent cleavage by signal peptidase (21). On day 0, 293 cells were set up for experiments as described under “Experimental Procedures.” Aliquots of DNA in each dish were adjusted to 2 μg/dish pCMV-SCAP (lanes 3 and 4). The total amount of DNA in each dish was adjusted to 2 μg by addition of pcDNA3 empty vector. After transfection, all cells were incubated in medium B for 23 h in the presence of sterols as described under “Experimental Procedures.” Aliquots of the membrane fraction (10 μg) were subjected to SDS-PAGE and immunoblot analysis with 5 μg/ml IgG-1C6 (anti-SREBP-2). Filters were exposed to film for 10 s.

main (Fig. 2A, lane 1). The mobility increased after treatment with peptide N-glycosidase F (lane 2) or endoglycosidase H (lane 3), but not with neuraminidase (lane 4). This pattern is consistent with the presence of N-linked carbohydrate chains that remained in the endoglycosidase H-sensitive form, indicating retention of the protein in the ER.

We also prepared a cDNA encoding a version of the glycosylated PSS/BP2 fusion protein that terminated at position 587 of SREBP-2, thereby eliminating the entire COOH-terminal regulatory domain (Fig. 2B). The truncated protein was visualized on SDS-PAGE by blotting with an antibody against the FLAG epitope. The truncated protein had an apparent molecular mass of 18 kDa (Fig. 2B, lane 1), and this was reduced by treatment with peptide N-glycanase F and endoglycosidase H, but not neuraminidase (lanes 2–4), indicating that it, too, was inserted into the ER and retained there.

Fig. 3 shows an experiment in which we tested the ability of PSS/BP2 fusion proteins to inhibit competitively the cleavage of full-length SREBP-2. To follow the fate of the full-length SREBP-2 in the transfected cells, we inserted an HSV epitope tag into the full-length sequence. The cells were incubated in the absence of sterols to induce cleavage at Site-1. When the HSV-SREBP-2 construct was transfected alone, nuclear extracts contained the NH2-terminal fragment, which was visualized by blotting with an anti-HSV tag antibody (Fig. 3, lane 1). On day 2, the cells were transfected with the indicated cDNAs as follows: 1 μg/dish wild-type (lanes 2 and 3) or R519A mutant version (lane 4) of pCMV-PSS/BP2-(504–1141) and 1 μg/dish pCMV-SCAP (lanes 3 and 4). The mobility increased after treatment with peptide N-glycosidase F (lane 4), indicating that it, too, was inserted into the ER and retained there.

Inhibition was also observed when the PSS/BP2-(504–1141) protein contained the R519A mutation (lane 9). However, no inhibition was seen when we expressed the truncated PSS/BP2-(504–587) protein (lane 10). We also blotted the membrane fraction with the anti-HSV tag antibody, and this confirmed that the transfected cells were all expressing the HSV-SREBP-2 protein in its precursor form. We blotted the membranes with an antibody against the FLAG epitope, and this confirmed that the expression of the PSS/BP2-(504–587) protein was at least as great as that of the PSS/BP2-(504–1141) version (Fig. 3B).

As reviewed in the Introduction, previous experiments have shown: 1) that the COOH-terminal regulatory domain of SREBP-2 is required for the protein to be cleaved at Site-1, and 2) that this segment interacts with the COOH-terminal WD repeat domain of SCAP to form an immunoprecipitable complex. We therefore interpreted the results of the experiment of Fig. 3 to suggest that the PSS/BP2-(504–1141) protein blocked cleavage of SREBP-2 because its COOH-terminal domain competed with SREBP-2 for binding to SCAP. If this is true, then overexpression of SCAP should reverse the inhibition by the PSS/BP2-(504–1141) protein. To test this hypothesis, we performed the experiment shown in Fig. 4. The 293 cells were transfected with the cDNA encoding HSV-SREBP-2, and again we observed the NH2-terminal fragment in nuclear extracts (lane 2). This was abolished by cotransfection with the cDNA
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FIG. 3. Prolactin signal sequence/SREBP-2 fusion protein inhibits cleavage of SREBP-2 in transfected 293 cells. The diagram shows the structure of the P450-TM/BP2-(555–1141) fusion protein in which the NH2-terminal 1–29 amino acids of cytochrome P450 2C1 are fused to the COOH-terminal domain of SREBP-2 (amino acids 555–1141). 293 cells were set up and transfected with 4 µg/dish pTK-HSV-BP2 (lanes 2–10) and the indicated amount of one of the following cDNAs: pCMV-PSS/BP2-(504–1141) (lanes 3–8), pCMV-PSS/BP2-(504–1141, R519A) (lane 9), or pCMV-PSS/BP2-(504–587) (lane 10). The total amount of DNA added to each dish was adjusted to 8 µg by addition of either pTK empty vector (5) or pcDNA3 empty vector. After transfection, all cells were incubated in medium B in the absence of sterols. On day 3, the cells were harvested and fractionated as described under "Experimental Procedures." 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 anti-FLAG M2 IgG for the prolactin signal sequence/SREBP-2 fusion protein (1). Filters were exposed to film for 20 s (nuclear extracts) and 20 s (membranes). The total amount of DNA in each dish was adjusted to 8 µg as described in Fig. 3. After transfection, all cells were incubated in medium B in the absence of sterols. On day 3, the cells were harvested and fractionated as described under "Experimental Procedures." 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 anti-FLAG M2 IgG for the prolactin signal sequence/SREBP-2 fusion protein (1). Filters were exposed to film for 20 s (nuclear extracts) and 20 s (membranes). N and P denote the cleaved nuclear and the uncleaved precursor forms of epitope-tagged SREBP-2, respectively. The circled numbers at the top of the figure refer to different cDNAs.

FIG. 4. SCAP overcomes inhibition of SREBP-2 cleavage mediated by prolactin signal sequence/SREBP-2 fusion protein in transfected 293 cells. The diagram shows the structure of the P450-TM/BP2-(555–1141) fusion protein in which the NH2-terminal 1–29 amino acids of cytochrome P450 2C1 are fused to the COOH-terminal domain of SREBP-2 (amino acids 555–1141). 293 cells were set up and transfected with 4 µg/dish pTK-HSV-BP2 (lanes 2–11), pCMV-PSS/BP2-(504–1141) (lanes 3–7), or pCMV-SCAP (lanes 4–7 and 8–11). The total amount of DNA in each dish was adjusted to 8 µg as described in Fig. 3. After transfection, all cells were incubated in medium B in the absence of sterols. Aliquots of nuclear extracts (60 µg of protein) and membranes (80 µg) were subjected to SDS-PAGE and immunoblotted with 0.5 µg/ml IgG-HSV-Tag™ for epitope-tagged SREBP-2. Filters were exposed to film for 20 s (nuclear extracts) and 20 s (membranes). N and P denote the cleaved nuclear and the uncleaved precursor forms of epitope-tagged SREBP-2, respectively. The circled numbers refer to different cDNAs.

FIG. 5. P450-TM/BP2-(555–1141) fusion protein inhibits cleavage of SREBP-2 in transfected 293 cells. The diagram shows the structure of the P450-TM/BP2-(555–1141) fusion protein in which the NH2-terminal 1–29 amino acids of cytochrome P450 2C1 are fused to the COOH-terminal domain of SREBP-2 (amino acids 555–1141). 293 cells were set up and transfected with 4 µg/dish pTK-HSV-BP2 (lanes 2–9) and the indicated amount of one of the following cDNAs: pCMV-P450-TM/BP2-(555–1141) (lanes 3–8), or pCMV-P450-TM/Gal (lane 9). The total amount of DNA in each dish was adjusted to 8 µg as described in Fig. 3. After transfection, all cells were incubated in medium B in the absence of sterols. On day 3, the cells were harvested and fractionated as described under "Experimental Procedures." Aliquots of nuclear extract (80 µg of protein) and membranes (80 µg) were subjected to SDS-PAGE and immunoblot analysis with IgG-HSV-Tag™ for epitope-tagged SREBP-2. Filters were exposed to film for 30 s (nuclear extracts) and 20 s (membranes). N and P denote the cleaved nuclear and the uncleaved precursor forms of epitope-tagged SREBP-2, respectively. The circled numbers refer to different cDNAs.

The experiments of Figs. 3 and 4 are consistent with the hypothesis that the PSS/BP2-(504–1141) protein inhibits cleavage of SREBP-2 because its COOH-terminal regulatory domain competes with this domain of SREBP-2 for interaction with SCAP. To test this hypothesis in a different way, we prepared a cDNA that encodes only the COOH-terminal regulatory domain of SREBP-2 without the lumenal loop or the second transmembrane domain (Fig. 5). To attach the COOH-terminal domain to membranes, we fused it to amino acids 1–29 of cytochrome P450–2C1. This segment was shown previously to cause the attachment of heterologous proteins to the cytoplasmic side of ER membranes (17). Transfection with increasing amounts of P450-TM/BP2-(555–1141) progressively reduced the amount of the NH2-terminal fragment of HSV-tagged SREBP-2 that appeared in nuclear extracts (lanes 3–8). As a control, we transfected a cDNA encoding the P450–2C1 NH2-terminal domain fused to β-galactosidase (17). This plasmid did not inhibit cleavage of HSV-tagged SREBP-2 (lane 9).

The inhibitory effect of P450-TM/BP2-(555–1141) on cleavage of HSV-SREBP-2 was reversed by co-transfection of a plasmid encoding wild-type SCAP (Fig. 6, lanes 4–7). SCAP did not encoding PSS/BP2-(504–1141) (lane 3). The inhibition was reversed when we cotransfected increasing amounts of a cDNA encoding wild-type SCAP (lanes 4–7). The SCAP cDNA produced only a slight stimulation when transfected in the absence of the PSS/BP2-(504–1141) fusion protein (lanes 8–11). As before, blotting of the membrane fractions with anti-HSV tag confirmed that the transfected cells were all expressing equal amounts of the HSV-SREBP-2 precursor.

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have any effect when it was transfected with the control plasmid encoding P450-TM/Gal (lanes 8–12).

If cleavage of SREBP-2 requires interaction of the COOH-terminal domains of SREBP-2 and SCAP, then overexpression of a membrane-anchored COOH-terminal domain of SCAP should also inhibit cleavage by competing with full-length SCAP for binding to SREBP-2. To test this hypothesis, we prepared a plasmid encoding the COOH-terminal WD repeat domain of SCAP fused to the P450–2C1 membrane anchor domain (Fig. 7). As shown previously, transfection of a plasmid encoding HSV-tagged SREBP-2 led to the buildup of the NH2-terminal domain (Fig. 7). As shown previously, transfection of a plasmid encoding HSV-tagged SREBP-2 led to the buildup of the NH2-terminal domain (Fig. 7). As shown previously, transfection of a plasmid encoding HSV-tagged SREBP-2 led to the buildup of the NH2-terminal domain (Fig. 7). As shown previously, transfection of a plasmid encoding HSV-tagged SREBP-2 led to the buildup of the NH2-terminal domain (Fig. 7). As shown previously, transfection of a plasmid encoding HSV-tagged SREBP-2 led to the buildup of the NH2-terminal domain (Fig. 7). As shown previously, transfection of a plasmid encoding HSV-tagged SREBP-2 led to the buildup of the NH2-terminal domain (Fig. 7).

Fig. 8A (top panel) shows a co-immunoprecipitation experiment that we designed as a direct test of the hypothesis that the truncated COOH-terminal domains of SREBP-2 and SCAP block the interaction of full-length SREBP-2 and SCAP. For this purpose, 293 cells were transfected with a cDNA encoding HSV-tagged full-length SREBP-2 under control of the TK promoter, which gives a relatively low level of expression. Cell extracts were incubated with an antibody to SCAP, and the immunoprecipitates were subjected to SDS-PAGE and blotted with an anti-HSV tag antibody that visualized the full-length SREBP-2 precursor (lane 2), indicating that the HSV-SREBP-2 formed a complex with endogenous SCAP. The HSV-SREBP-2 was not co-immunoprecipitated when we expressed an excess of P450-TM/BP2-(555–1141) (lane 3) or P450-TM/SCAP-(731–1276) (lane 4). Co-immunoprecipitation was also blocked by overexpression of the chimeric prolactin signal sequence construct (lane 5), but not the PSS/BP2-(504–587) version that terminated at position 587, which deletes the COOH-terminal domain of SREBP-2 (lane 6). The supernatants from all of the immunoprecipitations contained abundant full-length HSV-SREBP-2 precursor (Fig. 8A, bottom panel), indicating that the truncated proteins did not interfere with the synthesis of the full-length HSV-SREBP-2 precursor. Fig. 8B shows a control immunoblot, which demonstrates that similar amounts of endogenous SCAP were present in all of the immunoprecipitates.

To confirm that the COOH-terminal domains of SREBP-2 and SCAP inhibit SREBP-2 processing specifically at cleavage Site-1, we performed an experiment in M19 cells (Fig. 9). M19 cells are a mutant line of CHO cells that lack the gene encoding S2P, the putative Site-2 protease (2, 20). These cells carry out Site-1 cleavage, but the NH2-terminal domain of SREBP-2 remains in its membrane-bound intermediate form. When M19 cells were transfected with a cDNA encoding HSV-tagged SREBP-2 and incubated in the absence of sterols, the membrane fraction contained this intermediate form, which could be visualized by blotting with an antibody to the HSV tag (Fig. 9, lane 2). The presence of this fragment was abolished when the cells were cotransfected with plasmids encoding PSS/BP2-(504–1141) (lane 3) or P450-TM/SCAP-(731–1276) (lane 5). In both cases the inhibition was reversed by co-transfection of a cDNA encoding full-length SCAP (lanes 4 and 6).

Fig. 10 shows an experiment designed to test whether the inhibition of Site-1 cleavage of SREBP's has functional consequences for expression of SREBP target genes. For this purpose, we transfected 293 cells with a reporter construct consisting of a luciferase cDNA that was transcribed from a promoter that contains three copies of the SRE-1 element. As shown in Fig. 10A, this construct gave rise to abundant luciferase activity. The amount of luciferase was markedly reduced when we cotransfected increasing amounts of plasmids encoding P450-TM/BP2-(555–1141) or P450-TM/SCAP-(731–1276). The inhibition by both plasmids was reversed when we cotransfected...
Cleavage of SREBP-2 Requires SREBP-SCAP Complex

**Fig. 8.** Fusion proteins containing COOH-terminal domain of SREBP-2 or SCAP disrupt immunoprecipitable complexes between SREBP-2 and SCAP in transfected 293 cells. 293 cells were set up and transfected with one or more of the following cDNAs, as indicated in the figure: 3 μg/dish pTK-HSV-BP2 (lanes 2–6), 1.5 μg/dish pCMV-P450-TM/BP2 (555–1141) (lane 3), 1.5 μg/dish pCMV-P450-TM/SCAP (731–1276) (lane 4), 1.5 μg/dish pCMV-PSS/PPS-BP2 (504–1141) (lane 5), and 1.5 μg/dish pCMV-PSS/PPS-BP2 (504–587) (lane 6). The total amount of DNA in all dishes was adjusted to 6.5 μg as described in Fig. 3. After transfection, all cells were incubated in medium B in the presence of sterols. On day 3, the cells were harvested, and detergent-solubilized cell extracts were subjected to immunoprecipitation with 30 μg/ml polyclonal anti-SCAP IgG-R139 as described previously (4). The pellet (0.8 dis) and supernatant fractions (0.4 dis) from the immunoprecipitates were subjected to SDS-PAGE and immunoblotted with 0.5 μg/ml IgG-HSV-Tag™ for epitope-tagged SREBP-2 (A) or 5 μg/ml IgG-R139 for SCAP (B). The filters were exposed to film for 20 s (A) and 1 s (B). IP, immunoprecipitate.

increasing amounts of a plasmid encoding full-length SCAP (Fig. 10B).

**DISCUSSION**

The current experiments provide the first evidence that formation of an SREBP-2/SCAP complex is necessary in order for SREBP-2 to be cleaved at Site-1. Overexpression of truncated COOH-terminal domains of SREBP-2 or SCAP disrupted the SREBP-2/SCAP complex as measured by co-immunoprecipitation (Fig. 8). Under these conditions, SREBP-2 failed to be cleaved at Site-1 (Figs. 3–7 and 9). Cleavage was restored by overexpression of full-length SCAP (Figs. 4, 6, 7, and 9). The failure of SREBP cleavage led to a decreased expression of SRE-dependent genes, and this, too, was reversed by overexpression of SCAP (Fig. 10).

Fig. 11 shows a diagram that illustrates our interpretation of these results. As shown in previous co-immunoprecipitation experiments (4), SREBP-2 and SCAP form a complex by virtue of an interaction between their respective cytoplasmic COOH-terminal domains. We hypothesize that this complex recruits an enzyme, still undefined, which we call the Site-1 protease. The active site of this enzyme must be located in the lumen of the ER to cleave SREBP-2 at leucine 522. We envision the Site-1 protease as being an intrinsic membrane protein, but it might also be a soluble luminal protease that is bound extrinsically to the membrane, perhaps by interaction with SCAP. Overexpression of the truncated COOH-terminal domain of SCAP overcomes inhibition of SREBP-2 cleavage mediated by fusion proteins containing COOH-terminal domains of SREBP-2 or SCAP in transfected M19 cells. On day 0, M19 cells were set up at a density of 7 × 10⁶ cells/60-mm dish in medium C (a 1:1 mixture of Ham’s F12 medium and Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate) supplemented with 5% lipoprotein-deficient calf serum, 5 μg/ml cholest erol, 1 m mM sodium mevalonate, and 20 μM sodium olate. On day 1, cells were transfected with indicated plasmids using LipofectAMINE™ reagent (Life Technologies) as described previously (20) with the indicated amount of the following cDNAs: pTK-HSV-BP2 (lanes 2–6), pCMV-PSS/PPS-BP2 (504–1141) (lanes 3 and 4), pCMV-P450-TM/SCAP (731–1276) (lanes 5 and 6), and pCMV-SCAP (lanes 4 and 6). The total amount of DNA in each dish was adjusted to 5 μg as described in Fig. 3. After transfection, the cells were switched to medium D (medium C containing 5% newborn calf lipoprotein-deficient serum, 50 μM compain and 50 μM sodium mevalonate) in the absence of sterols. Aliquots of membrane fractions (60 μg of protein) were subjected to SDS-PAGE and immunoblotted with 0.5 μg/ml IgG-HSV-Tag™ for epitope-tagged SREBP-2. Filters were exposed to film for 20 s (I) and (P) denote the intermediate and the uncleaved precursor forms of epitope-tagged SREBP-2, respectively. The circled numbers refer to different cDNAs.

**Fig. 9.** Transfected P450-TM/BP2 (555–1141) and P450-TM/SCAP (731–1276) fusion proteins inhibit transcription of endogenous SREBP-driven luciferase reporter gene in 293 cells (A) and prevention of inhibition by transfected SCAP (B). A, 293 cells were cotransfected with the indicated amount of either pCMV-P450-TM/BP2 (555–1141) (●) or pCMV-P450-TM/SCAP (731–1276) (○) together with 0.2 μg/dish pSRE-Luc and 0.05 μg/dish pCMVβ-gal as described under “Experimental Procedures.” B, 293 cells were cotransfected with the indicated amount of pCMV-SCAP, 0.2 μg/dish pSRE-Luc, and 0.05 μg/dish pCMVβ-gal in the presence of either 0.25 μg/dish P450-TM/BP2 (555–1141) (●) or 0.1 μg/dish P450-TM/SCAP (555–1141) (○) as described under “Experimental Procedures.” A and B, the total amount of DNA in each transfection was adjusted to 1.35 μg/dish by addition of pcDNAs empty vector. After transfection, the cells were cultured in medium B in the absence of sterols. After incubation for 20 h, the cells were harvested, and luciferase activity was measured and normalized to β-galactosidase activity. Each value represents the average of duplicate incubations. The closed square in A denotes the luciferase activity of endogenous SREBP-2 in cells cultured in the presence of sterols.

**Fig. 10.** Transfected P450-TM/BP2 (555–1141) and P450-TM/SCAP (731–1276) fusion proteins inhibit transcription of endogenous SREBP-driven luciferase reporter gene in 293 cells (A) and prevention of inhibition by transfected SCAP (B). A, 293 cells were cotransfected with the indicated amount of either pCMV-P450-TM/BP2 (555–1141) (●) or pCMV-P450-TM/SCAP (731–1276) (○) together with 0.2 μg/dish pSRE-Luc and 0.05 μg/dish pCMVβ-gal as described under “Experimental Procedures.” B, 293 cells were cotransfected with the indicated amount of pCMV-SCAP, 0.2 μg/dish pSRE-Luc, and 0.05 μg/dish pCMVβ-gal in the presence of either 0.25 μg/dish P450-TM/BP2 (555–1141) (●) or 0.1 μg/dish P450-TM/SCAP (555–1141) (○) as described under “Experimental Procedures.” A and B, the total amount of DNA in each transfection was adjusted to 1.35 μg/dish by addition of pcDNAs empty vector. After transfection, the cells were cultured in medium B in the absence of sterols. After incubation for 20 h, the cells were harvested, and luciferase activity was measured and normalized to β-galactosidase activity. Each value represents the average of duplicate incubations. The closed square in A denotes the luciferase activity of endogenous SREBP-2 in cells cultured in the presence of sterols.
SREBP-2 or SCAP disrupts this complex by tying up full-length SCAP or SREBP-2, respectively. This prevents the Site-1 protease from accessing SREBP-2, thereby blocking cleavage. The cleavage can be restored by overexpression of full-length SCAP, which titrates out the inhibitor and restores SCAP-SREBP-2 complexes.

Although the current experiments were all performed with SREBP-2, we believe that the results are also applicable to SREBP-1a, at least in cultured cells. This is based on the data of Fig. 10, which shows that overexpression of truncated COOH-terminal domains of SREBP-2 or SCAP caused a near-complete block in the transcription of a reporter gene driven by an SRE-containing promoter. This implies that the action of endogenous SREBP-1a, as well as SREBP-2, is abolished by the truncated proteins.

The model in Fig. 11 is a minimalist model. It is not meant to exclude the possibility that other proteins are part of the SREBP-2-SCAP-Site-1 protease complex. We also do not mean to imply a 1:1 stoichiometry. Indeed, it is possible that multiple SCAP molecules interact with a single SREBP or vice versa. It should also be noted that sterols do not disrupt the SREBP-2-SCAP complex (4) even though they inhibit cleavage at Site-1. We believe that sterols interact with the polytopic membranous domain of SCAP, which resembles other proteins that are believed to interact with sterols (see the Introduction). This interaction may lead to inactivation of Site-1 protease, perhaps by displacing it from the complex, which otherwise remains intact.

In previous studies, we showed that the entire NH2-terminal basic-helix-loop-helix-leucine zipper domain of SREBP-2 can be replaced with an irrelevant protein such as Ha-Ras without affecting sterol-regulated cleavage at Site-1 (2). Here, we extend this finding by showing that the first transmembrane segment of SREBP-2 is also not required. Thus, the entire NH2-terminal and first transmembrane domains of SREBP-2 could be replaced with the prolactin signal sequence without abolishing cleavage at Site-1 (Fig. 1). We also recently showed that replacement of the second transmembrane domain of SREBP-2 with the membrane-spanning domain of the LDL receptor did not affect sterol-regulated cleavage at Site-1 (3). These findings localize the required domains of SREBP-2 to the lumenal loop and the COOH-terminal regulatory domain.

It is noteworthy that the truncated PSS/BP2-(504–587) protein failed to inhibit cleavage of full-length SREBP-2 (Fig. 3) even though a glycosylatable version of this protein underwent proper glycosylation (Fig. 2), indicating that the RSVL target sequence was present in the ER lumen. This finding implies that the RSVL sequence cannot interact with the active site of the protease unless it is attached to the COOH-terminal regulatory domain. One possible explanation is that the COOH-terminal SREBP-SCAP interaction delivers SREBP to the compartment where the protease is located. This site may be within the ER or it might be in a more distal compartment such as the Golgi complex. We are currently conducting pulse-chase studies to determine precisely the cellular compartment in which Site-1 cleavage occurs.

Full confirmation of the model in Fig. 11 awaits the identification and characterization of the Site-1 protease. We have been unsuccessful so far in identifying this enzyme through the use of in vitro biochemical assays designed to measure the cleavage of SREBP-2 or peptides containing the lumenal RSVL recognition sequence. Additional approaches are under way in which we are attempting to purify the proposed SREBP-2-SCAP-Site-1 protease complex. A somatic cell genetic approach is also being pursued.

One practical result of the current studies is that they provide a new way to create dominant negative proteins that block the activation of transcription by SREBPs. It should be possible to block SREBP action in tissue culture cells and in organs of living animals through use of vectors that overexpress truncated COOH-terminal domains of SREBP-2 or SCAP.

Acknowledgments—We thank Mark Daris for expert technical assistance; our colleagues Guoqing Cao, Elizabeth Duncan, and Dong Cheng for helpful discussions; Lisa Beatty and Lee Fowler for invaluable assistance; our colleagues Guoqing Cao, Elizabeth Duncan, and Dong Cheng for helpful discussions; Lisa Beatty and Lee Fowler for invaluable assistance; Jeff Cormier and Michelle Laremore for efficient DNA sequencing and oligonucleotide synthesis; and Dr. Shaun Coughlin and Dr. Byron Kemper for kindly providing plasmid constructs.

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