Identification of Complexes between the COOH-terminal Domains of Sterol Regulatory Element-binding Proteins (SREBPs) and SREBP Cleavage-Activating Protein*

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SREBP cleavage-activating protein (SCAP) stimulates the proteolytic cleavage of membrane-bound SREBPs, thereby initiating the release of NH2-terminal fragments from cell membranes. The liberated fragments enter the nucleus and stimulate transcription of genes involved in synthesis and uptake of cholesterol and fatty acids. Sterols repress cleavage of SREBPs, apparently by interacting with the membrane attachment domain of SCAP. In the present studies we show that SCAP, like the SREBPs, is located in membranes of the endoplasmic reticulum and nuclear envelope. The COOH-terminal domain of SCAP, like that of the SREBPs, is located on the cytosolic face of the membranes. Co-immunoprecipitation experiments show that SCAP and SREBP-2 form a complex that can be precipitated with antibodies to either component. Complex formation occurs when cells express only the COOH-terminal domain of either SREBP-2 or SCAP, indicating that the complex forms between the two COOH-terminal domains. Truncation of SREBP-2 at its COOH terminus prevents the formation of complexes with SCAP and simultaneously reduces proteolytic cleavage. We conclude that proteolytic cleavage of SREBPs requires the formation of a complex with the COOH-terminal domain of SCAP and that SCAP is therefore a required element in the regulation of sterol and fatty acid metabolism in animal cells.

Sterol regulatory element-binding proteins (SREBPs) are membrane-bound transcription factors that regulate the synthesis and uptake of cholesterol and fatty acids in animal cells (reviewed in Ref. 1). Two SREBPs, designated SREBP-1a and SREBP-2, predominate in cultured cells. The activities of both SREBPs are regulated by the sterol content of the cells. When cells are replete with sterols, the SREBPs remain bound to membranes of the endoplasmic reticulum (ER) and nuclear envelope and are therefore inactive. When cells are depleted of sterols, a proteolytic process releases the active portions of the SREBPs, which enter the nucleus and stimulate transcription of genes in three pathways of lipid metabolism: 1) cholesterol biosynthesis (HMG-CoA synthase, HMG-CoA reductase, farnesyl diphosphate synthase, and squalene synthase) (1–4); 2) fatty acid biosynthesis (acyetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase-1) (1, 5–7). The Site-1 enzyme requires the sequence DRSR, which is abolished. Cleavage at Site-2 also declines since this cleavage requires prior cutting at Site-1. As a result, the NH2-terminal segments remain bound to membranes, and transcription of the lipid-related genes is diminished.

The biochemical identities of the proteases that cut at Sites-1 and -2 are unknown, but certain requirements for substrate recognition have been elucidated. The Site-1 enzyme requires an arginine that is three residues to the NH2-terminal side of the cleaved Leu-Ser bond (9, 10). Even though it cuts on the luminal side of the membrane, the Site-1 enzyme requires the cytoplasmic COOH-terminal domain of the SREBP. When this domain in SREBP-2 is shortened through truncation mutations, cleavage by the Site-1 protease is abolished.2 The Site-2 enzyme requires the sequence DSVLSS, which is immediately external to the first transmembrane domain (10).

Recently, our laboratory described a new protein designated SREBP cleavage-activating protein (SCAP) that appears to mediate sterol regulation of cleavage at Site-1 (13). A cDNA lipoprotein receptor and lipoprotein lipase (1, 5); and 3) fatty acid biosynthesis (acyetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase-1) (1, 5–7).

2 J. Sakai, M. S. Brown, and J. L. Goldstein, unpublished observations.
encoding a sterol-resistant mutant form of SCAP was isolated by expression cloning from a line of CHO cells with a dominant defect in sterol regulation. In these cells sterols cannot suppress cleavage of SREBPs at Site-1, and thus the cells overproduce cholesterol. Expression cloning traced this defect to a substitution of asparagine for aspartic acid at residue 443 of SCAP. When a cDNA encoding the D443N mutant of SCAP is transfected into normal cells, the cells show increased cleavage of SREBPs at Site-1, and sterols no longer down-regulate this cleavage. Overexpression of a cDNA encoding wild-type SCAP can exert a similar effect, but the D443N mutant is at least 10-fold more potent (13). We concluded from these data that SCAP normally stimulates cleavage at Site-1 and that its activity is abolished by sterols. The mutant SCAP is both superactive and resistant to inhibition by sterols.

The hypothesis regarding the function of SCAP is supported by an analysis of the sequence of the 1276-amino acid protein, which reveals that SCAP is divided into two domains. The NH2-terminal domain of ~730 amino acids consists of alternating hydrophobic and hydrophilic segments that are compatible with approximately eight membrane-spanning domains (13). This is followed by a COOH-terminal domain of ~546 amino acids that is more hydrophilic and contains four or five WD repeats of ~40 amino acids each. WD repeats are found in more than 30 intracellular proteins of diverse function, including the β subunits of heterotrimeric G proteins (14). The latter proteins contain seven WD repeats that are clustered together to form a seven-bladed propeller-like structure, the blades of which contain seven WD repeats that are clustered together to form a membrane domain to the membrane domain of HMG-CoA reductase, an ER enzyme involved in cholesterol synthesis (13). These similarities led us to postulate that SCAP, HMG-CoA reductase is divided into two portions (17). The catalytic domain of the enzyme (17). The membrane domain is responsible for sterol-regulated degradation of the enzyme (19, 20). In sterol-depleted CHO cells, the enzyme has a long half-life of greater than 10 h. When sterols are added, the enzyme is destroyed with a half-life of ~1.5 h (19). Rapid degradation requires the membrane domain of the enzyme, which has been postulated to have a sterol-sensing function (19).

The membrane domain of SCAP contains a region (postulated membrane-spanning segments 2–6) that shows 25% sequence identity and 55% similarity to the corresponding portion of the sterol-sensing region of HMG-CoA reductase. This region includes aspartic acid 443, which is the site of the activating hydrophobic and hydrophilic segments that are compatible with approximately eight membrane-spanning domains (13). These similarities led us to postulate that the membrane domain of SCAP, like that of HMG-CoA reductase, is a sterol sensor. There is no evidence that sterols accelerate the degradation of SCAP, as they do of HMG-CoA reductase. Instead, sterols may interact with the membrane domain of SCAP so as to regulate its ability to stimulate the cleavage of SREBPs at Site-1 (1).

In the current studies we have sought to further understand the mechanism by which SCAP may regulate the cleavage of SREBPs. Through use of a co-immunoprecipitation assay, we show that the COOH-terminal cytoplasmic domain of SREBP-2 forms a complex with the COOH-terminal WD repeat domain of SCAP. We postulate that this interaction allows SCAP to recruit a protease that cleaves SREBPs at Site-1. This hypothesis would explain the finding that cleavage at Site-1 requires the COOH-terminal domain of SREBPs.

EXPERIMENTAL PROCEDURES

Materials—We obtained monoclonal antibodies IgG-HSV-Tag™ and IgG-T7-Tag™ from Novagen; v-Ha-Ras(Als1)-agarose-linked monoclonal antibody from Oncogene; affinity-purified donkey anti-mouse and anti-rabbit IgG from Jackson Immunoresearch Laboratories; Protein G-Sepharose® 4 Fast Flow beads from Pharmacia Biotech Inc. Other reagents were obtained from sources as described previously (8, 21).

Constructions of Plasmids—pTK-HSV-Ras-BP2 is an expression vector encoding an Ha-Ras/SREBP-2(473–1141) fusion protein with two NH2-terminal human HSV epitope tags under control of the HSV thymidine kinase promoter (10). pTK-HSV-BP2-Ras-T7 is an HSV thymidine kinase promoter-driven expression vector encoding an SREBP-2(14–1141)/Ha-Ras fusion protein flanked at the NH2 terminus by two tandem HSV epitope tags and at the COOH terminus by three tandem copies of an epitope derived from the T7 major capsid protein (9). pCMV-SCAP is a cytomegalovirus promoter-driven expression vector encoding hamster SCAP that is similar to pCMV-SCAP (13) except that the expression vector was switched from pRC/CMV75SB to pCDNA3 (Invitrogen).

The expression vector pCMV-HSV-BP2(555–1141) encodes amino acids 555–1141 of human SREBP-2 preceded by an initiator methionine, two tandem copies of the HSV epitope (QPELAPEDPED), and two novel amino acids (ID) encoded by a sequence for the BspDI restriction site. pCMV-HSV-BP2(555–1141) was constructed as follows. First, pCMV-HSV-BP2 (see Ref. 13) was digested with BspDI and PmlI, and the 7.0-kb BspDI-BspDI fragment containing the HSV epitopes and amino acids 970–1141 of SREBP-2 was isolated. Second, the sequence corresponding to amino acids 555–969 of human SREBP-2 was amplified by PCR (8) of pTK-HSV-BP2 (see Ref. 21) with an NH2-terminal primer flanked by a BspDI site and a COOH-terminal primer flanked by a PmlI site. The resulting 1.3-kb BspDI-PmlI fragment was ligated to the above 7.0-kb BspDI-BspDI fragment to generate pCMV-HSV-BP2(555–1141).

The expression vector pCMV-HSV-BP2(555–1141)-Ras-T7 encodes a fusion protein consisting of an initiator methionine, two tandem copies of the HSV epitope (QPELAPEDPED), two novel amino acids (ID) encoded by the restriction site for BspDI, amino acids 555–1141 of human SREBP-2, two novel amino acids (HM) encoded by the restriction site for NotI, amino acids 2–189 of human Ha-Ras, and three tandem copies of the T7 epitope (9). pCMV-HSV-BP2(555–1141)-Ras-T7 was constructed as follows. First, pCMV-HSV-BP2 (see Ref. 13) was digested with Apal to isolate a 7.3-kb fragment containing the HSV epitopes followed by human SREBP-2 (amino acids 14–505). pTK-HSV-BP2-Ras-T7 (see Ref. 9) was digested with Apal to isolate a 2.6-kb Apal-Apal fragment encoding a fusion protein consisting of human SREBP-2(14–505), human Ha-Ras(2–189), 21).

The expression vector pCMV-HSV-BP2(555–1141)-Ras-T7 encodes a fusion protein consisting of an initiator methionine, amino acids 732–1276 of hamster SCAP, and three tandem repeats of the T7 epitope. pCMV-SCAP(732–1276)-T7 was constructed as follows. First, the sequence corresponding to amino acids 732–910 of SCAP was amplified by PCR of pCMV-SCAP (13) with a primer of primers, 5′-ATACATGTTACAGTTTGCTTGCCCGCGCGAGCT-3′ (encoding amino acids 732–738 of SCAP preceded by an SpeI site and an initiator methionine) and 5′-GTTGGAAGTATCCAGGAGACT-3′ (encoding amino acids 904–910 of SCAP). The PCR fragment was cloned into the pNOta/T7 vector (v-Ha-Ras(Ab-1)-agarose-linked monoclonal antibody from Oncogene; affinity-purified donkey anti-mouse IgG-T7-Tag™ from Novagen; pCMV-SCAP). The PCR fragment was cloned into the pNOta/T7 vector (5 Prime-3 Prime, Inc.) and digested with BamHI, which cuts in the polylinker of the pNOta/T7 vector, and NotI to isolate a 0.57-kb fragment encoding amino acids 732–900 of SCAP. Second, pCMV-SCAP(732–1276) (provided by Tong Yang, University of Texas Southwestern Medical Center) was digested with BamHI and NotI, and the 0.38-kb fragment encoding amino acids 773–900 of SCAP was replaced with the BamHI-NotI 0.5-kb fragment encoding amino acids 732–900 (described above) to yield the resulting plasmid pCMV-SCAP(732–1276). Third, pTK-HSV-SREBP-2-T7 (see Ref. 13) was digested with Apal, and the resulting 1.0-kb fragment encoding amino acids 961–1276 of SCAP and three repeats of the T7 epitope was used to replace amino acids 961–1276 of SCAP in pCMV-SCAP(732–1276).
The structures of the above plasmids were confirmed by sequencing all PCR fragments and all ligation joints.

Site-directed Mutagenesis—Oligonucleotide mutagenesis was carried out with single-stranded uracil-containing DNA (22) using the Mutagen Phagemid kit (Bio-Rad) as described previously (8). Each mutant was confirmed by sequencing. The mutants of at least two independent clones of each mutant were independently transfected into 293 cells to confirm the results.

Antibodies—Polyclonal antibody IgG-R139 against hamster SCAP was produced by immunizing rabbits with a mixture of two fusion proteins, one encoding six consecutive histidines followed by amino acids 64–227 of SCAP and the other encoding six consecutive histidines followed by amino acids 540–707 of SCAP (13). Monoclonal antibody IgG-9D5 against hamster SCAP was produced by immunizing a mouse (23) with a fusion protein encoding six consecutive histidines followed by amino acids 540–707 of SCAP (13). The cDNAs encoding the histidine-tagged proteins were cloned into pET28a (+) vector (Novagen) and expressed in Escherichia coli, and the proteins were purified by Ni⁺²⁺-

Sepharose chromatography as described (24). Monoclonal antibodies IgG-2A4 against the basic-helix-loop-helix domain of human SREBP-1 (25), IgG-7D4 directed against hamster SREBP-2 (amino acids 32–250) (11), and IgG-1C6 directed against the COOH terminus of human SREBP-2 (amino acids 833–1141) (8) have been described in the indicated references. Other monoclonal antibodies were obtained commercially as described above.

Culture, Transfection, and Fractionation of 293 Cells—Monolayers of human embryonic kidney 293 cells were set up on day 0 (4 × 10²⁶ 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 the indicated plasmids as described previously (8). Three h after transfection, the cells were switched to medium B (medium A containing 10% newborn calf lipoprotein-deficient serum) (10). On day 1, cells were transfected with 5 μg of pcCMV-SCAP/dish (pcCMV-SCAP is a neo-containing expression vector encoding wild-type hamster SCAP, see above). On day 2, the medium was switched to medium B containing 700 μg/ml G418. The medium was changed every 2nd day until well defined colonies were evident on day 12–14. Colonies that were isolated with cloning cylinders in the presence of 150 μg/ml G418 were stored frozen for use for transient transfection experiments. The resulting stable cell lines were analyzed by immunoblotting with anti-SCAP IgG-9D5, and the highest expressing line was selected for immunofluorescence analysis.

Co-immunoprecipitation/Immunoblot Assay—Cells from 2 dishes of 293 cells were harvested, and the pooled cell pellet was solubilized with 1 ml of Nonidet P-40 lysis buffer (50 mM Hepes-SCAP at pH 7.4, 100 mM NaCl, 1.5 mM MgCl₂, 1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 25 μg/ml N-acetyl-leucinal-leucinal-norleucinal (ALLN), and 1 mM di-thiothreitol), passed through a 22.5-gauge needle 15 times, and extracted by rotating for 1.5 h at 4 °C. All subsequent operations were carried out at 4 °C unless otherwise stated. The cell extracts were clarified by centrifugation at 10³ × g for 30 min in a Beckman TLA 120.2 rotor. An aliquot of the supernatant (0.2–1.5 mg of protein in 0.2–0.9 ml) was adjusted to a final volume of 1 ml with Nonidet P-40 lysis buffer and preclariﬁed by rotation for 1 h with either 20 μg of an irrelevant mouse monoclonal antibody IgG-2001 (see Ref. 27) (for immunoprecipitation with monoclonal antibodies) or 20 μg of an IgG fraction of nonimmune rabbit serum (for immunoprecipitation with a polyclonal antibody) and 50 μl of Protein G-Sepharose beads. Rotation was performed in a Cole-Palmer instrument Co. apparatus (model 67367) at 15–20 rpm in a 4 °C cold room. After centrifugation at 300 × g for 3 min, the supernatant was transferred to a fresh tube, and immune monononal or polyclonal antibodies were added. After rotating for 1.5 h, 50 μl of Protein G-Sepharose beads were added, followed by rotation for 1.5–3 h, and centrifugation at 300 × g for 3 min. The resulting supernatant was transferred to a new tube and precipitated with five vol

![Fig. 1. Indirect immunofluorescence localization of wild-type SCAP in stably transfected CHO cells.](image)

Prior to SDS-PAGE, the immunoprecipitate and supernatant fractions each received 25 μl of 5 × buffer B. They were then boiled for 5 min and subjected to SDS-PAGE and immunoblot analysis. The proteins were transferred to Hybond-C extra nitrocellulose filters (Amersham Corp.), which were incubated with one of the antibodies described above. Bound antibodies were visualized with peroxidase-conjugated affinity-purified donkey anti-mouse or anti-rabbit IgG using the SuperSignal CL-HR substrate system (Pierce) according to the manufacturer’s instructions. Gels were calibrated with prestained molecular weight markers (New England Biolabs). Filters were exposed to Reflection™ NEF-496 film (NEN Life Science Products) at room temperature for the indicated time.

RESULTS

Previous studies demonstrated that the full-length precursor forms of the SREBPs are bound to membranes of the ER and nuclear envelope, as measured by immunofluorescence staining of transfected cells (25, 28). To determine whether SCAP is similarly localized, we prepared a line of CHO cells that permanently expresses elevated levels of wild-type SCAP as a stable transfectant (Fig. 1). The diagram at the top of Fig. 2 illustrates the proposed orientation of SREBP-2 and SCAP in ER membranes. The diagram also shows the postulated interaction between the COOH termini of the two proteins, both of which are oriented toward the cytoplasm. The proposed orientation of SREBP-2 is...
supported by previously published studies that employed protease protection (8) and insertion of sites for N-linked glycosylation into the luminal loop (8, 9). The placement of the COOH terminus of SCAP on the cytosolic face of the membrane is based on several lines of evidence: 1) protease protection experiments to be published elsewhere; 2) the structural resemblance of SCAP to HMG-CoA reductase, whose COOH-terminal domain faces the cytoplasm (17–19); and 3) the demonstration in the current paper that the COOH-terminal domain of SCAP interacts with the COOH-terminal domain of SREBP-2, which is known to face the cytoplasm (1). We show SCAP with eight membrane-spanning regions (13) by analogy to HMG-CoA reductase (18), but we have no direct evidence for the exact number, and hence the NH₂ terminus of SCAP might be on either side of the membrane.

Panels A and B of Fig. 2 illustrate the results of a co-immunoprecipitation assay designed to measure the interaction of endogenous SCAP with endogenous SREBP-1 and SREBP-2 in nontransfected CHO cells. Prior to the experiment, the cells were incubated in medium containing lipoprotein-deficient serum and 50 μg/ml cholesterol (+ Sterols), or both as indicated. All cultures were adjusted to contain 0.2% ethanol. On day 3, the cells were harvested, and detergent-solubilized cell extracts were subjected to immunoprecipitation with 30 μg of polyclonal anti-SCAP IgG-R139 as a control. The antibodies against SREBP-1 and -2 were directed against the NH₂-terminal domains and thus they visualized the proteolytically processed mature NH₂-terminal fragments of SREBP-1 and -2, which are localized in cell nuclei (bands denoted by M in Fig. 2). As expected, the amounts of these fragments were increased by incubation with compactin and decreased by sterols. Importantly, all of the NH₂-terminal fragments were found in the supernatants, and none was precipitated with the anti-SCAP antibody.

The experiment of Fig. 2 revealed another important facet of the SREBP-SCAP interaction. The amounts of SREBP-1 and -2 that were co-immunoprecipitated with SCAP did not change under conditions of sterol depletion or repletion, even though the rate of proteolytic processing varied dramatically under these conditions. Thus, sterols do not seem to regulate SREBP processing by regulating the interaction between SCAP and SREBPs.

To gain more insight into the mechanism of the SCAP interaction with SREBPs, we performed a series of experiments in human embryonic kidney 293 cells that were induced to express varying forms of epitope-tagged SREBPs or SCAP as a result of transient transfection. Fig. 3 shows one such experiment in which the cells were transfected with cDNAs encoding SCAP and/or a tagged version of SREBP-2 with an NH₂-terminal epitope tag consisting of two copies of a short peptide

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3 A. Nohturfft, J. Sakai, J. L. Goldstein, and M. S. Brown, manuscript in preparation.
We showed previously that overexpressed wild-type SCAP stimulates cleavage of SREBPs (13). The experiment shown in Fig. 4 was designed to determine whether this stimulation requires the COOH-terminal domain. The design of the experiment is based on three previous observations: 1) the NH$_2$-terminal domain of SREBPs can be replaced by an irrelevant sequence, such as the Ras protein, without disrupting sterol-regulated cleavage (10); 2) truncation of SREBP-2 at the COOH terminus progressively abolishes sterol-regulated cleavage (2); and 3) overexpression of wild-type SCAP stimulates cleavage of SREBPs, and it overcomes the suppressive effect of sterols (13). 293 cells were transfected with a cDNA encoding a chimeric form of SREBP-2 with an HSV-tagged Ras substituted for the COOH-terminal domain. The cells were incubated with sterols, and hence no HSV-Ras was released into the cytosol.

Fig. 3. Co-immunoprecipitation of epitope-tagged SREBP-2 and SCAP produced by transfection in 293 cells. The diagram shows the proposed sites of interaction of epitope-tagged SREBP-2 with SCAP. A and B, co-immunoprecipitation analysis. On day 0, 293 cells were set up for experiments as described under "Experimental Procedures." On day 2, the cells were transfected with the indicated plasmids as follows: 3 µg of pTK-HSV-BP2/dish (lanes 2, 3, 5, and 6), 1 µg of pCMV-SCAP/dish (lanes 3 and 6), and 2 µg of pVAI/dish (lanes 1–6). (pVAI encodes the adenovirus-associated I DNA gene, which enhances translation of mRNAs produced by transfected cDNAs; Ref. 31). The total amount of DNA was adjusted to 6 µg/dish by addition of either pTK mock vector (21) or pcDNA3 mock vector. After transfection, all cells were incubated in medium B in the presence of 1 µg/ml 25-hydroxycholesterol plus 10 µg/ml cholesterol. On day 3, the cells were harvested and subjected to immunoprecipitation with 30 µg/ml monoclonal anti-SCAP IgG-R139 as described under "Experimental Procedures." The pellet and supernant fractions from the immunoprecipitation of 0.2 dish of cells were subjected to SDS-PAGE and immunoblotted with 0.5 µg/ml IgG-HSV-Tag™ for epitope-tagged SREBP-2 (A) or 7.5 µg/ml IgG-9D5 for SCAP (B). The filters were exposed to film for 6 s (A) and 8 s (B). IP, immunoprecipitate; Sup., supernatant.

Fig. 4. SCAP-stimulated cleavage of Ha-Ras/SREBP-2 in transfected 293 cells depends on COOH-terminal domain of SREBP-2. The diagram shows epitope-tagged Ha-Ras/SREBP-2 fusion proteins containing the full-length COOH-terminal domain of SREBP-2 (STOP 1142) or two truncations (STOP 793 and STOP 588). 293 cells were set up for experiments and transfected with 3 µg of plasmid/dish (plasmid encoding the indicated version of pTK-HSV-Ras-BP2) (lanes 2–7), 1 µg of pCMV-SCAP/dish (lanes 3, 5, and 7), and 2 µg of pVAI/dish (lanes 1–7). The total amount of DNA was adjusted to 6 µg/dish as described in Fig. 3. After transfection, all cells were incubated in medium B in the presence of sterols as described in Fig. 3. On day 3, the cells were harvested, and cytosol and membrane fractions were prepared as described under "Experimental Procedures." Aliquots of membranes (140 µg) and cytosol (80 µg) were subjected to SDS-PAGE and immunoblot analysis with 0.5 µg/ml IgG-HSV-Tag™. Filters for membrane and cytosol fractions were exposed to film for 10 s. For the membrane fractions, the numbers on the right vertical axis denote the positions of migration of the uncleaved proteins. For the cytosol fractions, the arrow denotes the position of the cleaved HSV-Ras that is released into the cytosol.
SCAP, cleavage of the HSV-Ras/SREBP-2 fusion protein was stimulated, and the HSV-tagged Ras was found in the cytosol (lane 3). When the COOH terminus of the chimeric protein was truncated at residue 793 of SREBP-2, which corresponds to the end of exon 12 (29), SCAP still stimulated cleavage of the protein, but the degree of stimulation was markedly reduced (compare lane 5 with lane 3 in cytosol fraction). When the protein was further truncated at residue 588, which corresponds to the end of exon 9, SCAP no longer stimulated proteolytic cleavage (lanes 6 and 7). The STOP 588 protein retains only 33 amino acids following the second transmembrane domain. These data indicate that SCAP stimulates cleavage of SREBP-2 only when the COOH-terminal domain is present.

Fig. 5 shows that SCAP interacts with SREBP-2 only when the COOH terminus is present. 293 cells were transfected with a cDNA encoding full-length SCAP plus a cDNA encoding the HSV-Ras/SREBP-2 fusion protein that terminates at a position corresponding to the COOH terminus of wild-type SREBP-2 (STOP 1142) or at the premature truncation sites that were described in Fig. 4 (STOP 793 and STOP 588). To increase the amounts of the uncleaved precursors that are available for co-immunoprecipitation, we introduced the R519A mutation, which retards cleavage at Site-1 (10). In panel A of Fig. 5, the cell extracts were immunoprecipitated with anti-SCAP and blotted with the antibody against the HSV-tag at the NH2 terminus of the HSV-Ras/SREBP-2 fusion protein. In the SCAP-transfected cells, significant amounts of the STOP 1142 construct were co-immunoprecipitated with SCAP (lane 3), but we detected only trace amounts of the STOP 793 construct (lane 5) and none of the STOP 588 construct (lane 7). Panel B

| cDNA          | SCAP          | IP with Anti-SCAP | Blot with Anti-HSV-tag |
|---------------|---------------|-------------------|------------------------|
| HSV-Ras-BP2   |   | | |
| STOP 1142     |   | | |
| STOP 793      |   | | |
| STOP 588      |   | | |
| SCAP          |   | | |
| HSV-Ras-BP2   |   | | |
| STOP 1142     |   | | |
| STOP 793      |   | | |
| STOP 588      |   | | |
| SCAP          |   | | |

Fig. 5. Interaction of Ha-Ras/SREBP-2 and SCAP in transfected 293 cells depends on the COOH-terminal domain of SREBP-2. The diagram shows the proposed sites of interaction of transfected epitope-tagged Ha-Ras/SREBP-2 and transfected SCAP. The numbers in the COOH-terminal domain of Ras/SREBP-2 refer to the sites of truncation as described in Fig. 4. A-C, co-immunoprecipitation analysis. 293 cells were set up and transfected with 3 μg of plasmid/dish (encoding the indicated version of pTK-HSV-Ras-BP2) (lanes 2–7 and 9–14), 1 μg of pCMV-SCAP/dish (lanes 3, 5, 7, 10, 12, and 14), and 2 μg of pV5/His di/dish (lanes 1–14). The total amount of DNA was adjusted to 6 μg/dish as described in Fig. 3. After transfection, all cells were incubated in medium B in the presence of sterols as described in Fig. 3. On day 3, the cells were harvested, and detergent-solubilized cell extracts were subjected to immunoprecipitation with either 30 μg of polyclonal anti-SCAP IgG-R139 (A and B) or 3 μg of IgG-HSV-Tag™ plus 25 μg of α-Ha-Ras/Ab-1-agarose linked monoclonal antibody (C) as described under “Experimental Procedures.” The pellet and supernatant fractions from immunoprecipitation of 0.2 dish of cells were subjected to SDS-PAGE and immunoblotted with 0.5 μg/ml IgG-HSV-Tag™ (A), 7.5 μg/ml monoclonal anti-SCAP IgG-9D5 antibody (B), or 2.5 μg/ml polyclonal anti-SCAP IgG-R139 (C). The filters were exposed to film for 7 s (A), 2 s (B), or 6 s (C). IP, immunoprecipitate.
is a control immunoblot, which shows that similar amounts of SCAP were present in all three immunoprecipitates.

Panel C of Fig. 5 shows the results when the order of antibodies was reversed. In this case we precipitated with antibodies against the HSV-Ras/SREBP-2 fusion protein and blotted with anti-SCAP. Again, significant coimmunoprecipitation occurred only with the STOP 1142 construct (panel C, lane 3). We conclude from this experiment that the interaction of SCAP with SREBP-2 requires the COOH-terminal domain of SCAP (residues 732–1276) that includes only the COOH-terminal domain (Fig. 6). In the first part of the experiment (panel A), we produced the COOH-terminal fragment of SREBP-2 (residues 555–1141) with or without a COOH-terminal extension encoding full-length Ras plus three repeats of an epitope derived from a bacteriophage T7 protein (T7-tag). Cell extracts were immunoprecipitated with anti-SCAP and blotted with an antibody against the COOH-terminal domain of SREBP-2. The untagged COOH-terminal domain of SREBP-2 was found in the pellet, but only when SCAP was co-transfected (compare lanes 2 and 3). A similar result was obtained with the Ras-T7-tagged COOH-terminal fragment (compare lanes 4 and 5).

Panel B shows that SCAP was detected in the immunoprecipitate when the SCAP cDNA was co-transfected.

In panel C of Fig. 6, we reversed the order of immunoprecipitation. Cell extracts were precipitated with a combination of antibodies against Ras and the T7 epitope and blotted with anti-SCAP. SCAP was found in the pellet only when both cDNAs were transfected (panel C, lane 3). Panel D confirms that the tagged SREBP-2 fusion proteins were precipitated by the antibodies against the Ras and T7 tags.

Having demonstrated that the COOH-terminal domain of SREBP-2 mediates the interaction with SCAP, we next set about to identify the domain of SCAP that was required. For this purpose we transfected 293 cells with the cDNA encoding the HSV-Ras/SREBP-2 fusion protein plus a cDNA encoding the COOH-terminal domain of SCAP (residues 732–1276) that was tagged with three copies of the T7 epitope. Panel A of Fig. 7 shows the results when SCAP was immunoprecipitated with the antibody against the T7-tag and SREBP-2 was visualized by blotting with the antibody against the HSV-tag. The Ras/SREBP-2 fusion protein was immunoprecipitated, but only
when the COOH terminus of SCAP was expressed (compare lanes 2 and 3 of panel A). Panel B is a control immunoblot showing that the anti-T7 antibody precipitated the T7-tagged COOH-terminal fragment of SCAP.

To rule out the possibility that the SREBP-2-SCAP complexes were forming after the cells were solubilized, we performed a mixing experiment (data not shown). Two dishes of 293 cells were transfected with pCMV-SCAP, and two separate dishes were transfected with pTK-HSV-Ras-BP2 plus pVAI as described in legend to Fig. 3. Cell extracts from the two separate transfections were prepared, mixed together, and incubated for 5 h during the preclearing period and the immunoprecipitation with anti-SCAP. The pellet and supernatant fractions from immunoprecipitation with 3 μg of protein A-Sepharose and 2 μg of pVAI/dish (lanes 1–6). The total amount of DNA was adjusted to 6 μg/dish as described in Fig. 3. After transfection, all cells were incubated in medium B in the presence of the cDNAs for SCAP and Ras/SREBP-2.

DISCUSSION

The current results establish that SCAP forms a complex with the full-length precursor form of SREBP-2 in cultured cells and that formation of this complex correlates with the ability of SCAP to stimulate cleavage of SREBP-2 at the sterol-regulated site (Site-1). Complex formation is mediated by the COOH-terminal domains of SREBP-2 and SCAP, both of which are located on the cytosolic surface of the ER and nuclear envelope.

The conclusion that the SCAP-SREBP-2 interaction is necessary for Site-1 cleavage is based on the observation that truncations of SREBP-2 at positions that remove 349 or 554 amino acids from the COOH terminus progressively reduce Site-1 cleavage (Fig. 4) and simultaneously reduce the binding of SCAP (Fig. 5).

The interaction between SREBP-2 (or SREBP-1) and SCAP was observed in co-immunoprecipitation assays with nontransfected cells that express only the normal endogenous levels of these proteins (Fig. 2). It was also observed in extracts of transfected cells that overproduce both SCAP and SREBP-2 (Figs. 3 and 5). The complex could be precipitated with antibodies against SCAP (Figs. 2 and 3) or with antibodies against epitope-tagged SREBP-2 (Fig. 5C). The complex could also be precipitated when the transfected cells expressed only the COOH-terminal domain of either SCAP or SREBP-2 (Figs. 6 and 7), confirming that the COOH-terminal domains were responsible for the interaction. We believe that the complex forms by direct interaction between the COOH-terminal domains of SCAP and SREBP, but we cannot rule out the possibility that the two domains each interact with a third protein that bridges the complex. We also cannot rule out the possibility that the membranous domain of SCAP plays some role in the interaction with SREBPs, but this domain is clearly not essential for this interaction.

The involvement of the COOH-terminal domain of SCAP in this protein-protein interaction is consistent with the presence in this domain of at least four (and likely five) WD repeats (13). These repeat sequences have been shown crystallographically to mediate protein-protein interactions in heterotrimeric G proteins (15, 16), and they have been implicated biochemically in such interactions in several other proteins (14).

Although the SCAP-SREBP-2 interaction occurs on the cytoplasmic face of the membrane, it leads to cleavage of SREBP-2 at Site-1 on the lumenal side of the membrane. SCAP itself does not appear to be a protease, as indicated by its lack of sequence resemblance to known proteases and by its failure to cleave SREBPs in co-translation assays. It seems likely, therefore, that the SCAP-SREBP-2 complex forms a binding site for a third protein, a protease, whose active site faces the lumen of the ER. We are currently attempting to isolate sufficient amounts of the SCAP-SREBP-2 complex so as to permit detection of any additional components.

We do not know the fate of the SCAP-SREBP-2 complex after the SREBP-2 is cleaved. We have shown that the COOH-terminal fragment of SREBP-2 remains membrane-bound and is eventually degraded (8). It is possible that SCAP remains associated with this fragment and is degraded with it. Alternatively, SCAP may recycle by dissociating from the COOH-terminal fragment after cleavage, following which it may bind to another SREBP precursor molecule.

In untransfected cells about 20–40% of the SREBP-2 precursor was co-immunoprecipitated with SCAP, and the rest remained in the supernatant (Fig. 1). We believe that nearly all of the SCAP in the cell extract was precipitated, but technical problems prevent us from confirming this conclusion by showing a disappearance of SCAP from the supernatant. In the supernatant SCAP is very dilute, and the protein aggregates when we attempt to concentrate it prior to electrophoresis (see lanes 7 and 8 in Fig. 6C). The incomplete precipitation of SREBP-2 suggests that the amount of SCAP is rate-limiting in complex formation and that only 20–40% of the SREBP precursors are in a complex with SCAP at any one time. This hypothesis is supported by the observation that overexpression
of SCAP stimulates the cleavage of endogenous SREBPs (13), which implies that the amount of SCAP is ordinarily rate-limiting. We cannot rule out the alternate possibility that all of the cell’s SREBPs are in a complex with SCAP, but some of the complexes dissociate during detergent extraction, immunoprecipitation, or washing.

The experiment of Fig. 2 demonstrates that the amount of SCAP-SREBP-2 complex is not altered when SREBP-2 cleavage is stimulated by incubation with compaction, or inhibited by incubation with sterols. These data indicate that SCAP does not respond to sterols by dissociating from SREBP-2. Rather, sterols may cause SCAP to dissociate from an accompanying protease, rendering the SCAP-SREBP-2 complex incompetent to carry out proteolysis.

The experiment of Fig. 2 also shows that endogenous SREBP-1, as well as SREBP-2, is co-immunoprecipitated with an antibody against SCAP. All of the subsequent transfection experiments were performed with SREBP-2. We have recently performed similar experiments with transfected epitope-tagged SREBP-1a and have obtained similar results, namely that SREBP-1a forms a complex with SCAP and that the amount of this complex is markedly reduced when the COOH-terminal domain of SREBP-1a is truncated (data not shown).

We have shown previously that 25-RA cells produce a mutant form of SCAP(D443N), which is hyperactive in stimulating cleavage of SREBPs and which resists down-regulation by sterols (13). In experiments not shown, we have found that SCAP(D443N) is co-immunoprecipitated with SREBP-2 to the same extent as wild-type SCAP in transfected 293 cells in the presence of sterols. Thus, the D443N mutation does not alter the direct interaction of SREBPs and SCAP, but it must alter some other function that renders the complex a better substrate for the Site-1 protease.

The current results provide strong support to the notion that SCAP is a required component of the pathway that leads to sterol-regulated cleavage of SREBPs at Site-1. Studies are now under way to further test this hypothesis and to make use of the SCAP-SREBP-2 complex to isolate the sterol-regulated protease.

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