The polytopic membrane protein SCAP transports sterol regulatory element-binding proteins (SREBPs) from the endoplasmic reticulum (ER) to the Golgi, thereby activating cholesterol synthesis. Cholesterol accumulation in the ER membranes changes SCAP to an alternate conformation in which it binds ER retention proteins called Insigs, thereby terminating cholesterol synthesis. Here, we show that the conserved Asp-428 in the sixth transmembrane helix of SCAP is essential for SCAP’s dissociation from Insigs. In transfected hamster cells, mutant SCAP in which Asp-428 is replaced by alanine (D428A) remained in an Insig-binding conformation when cells were depleted of sterols. As a result, mutant SCAP failed to dissociate from Insigs, and it failed to carry SREBPs to the Golgi. These data identify an important functional residue in SCAP, and they provide genetic evidence that the conformation of SCAP dictates the rate of cholesterol synthesis in animal cells.

The interaction of two polytopic membrane proteins, SREBP cleavage-activating protein (SCAP) and Insig, is a central event in the control of cholesterol homeostasis in animal cells (1, 2). SCAP is an escort protein for sterol regulatory element-binding proteins (SREBPs) that are membrane-bound transcription factors activating genes encoding enzymes required for synthesis of cholesterol and other lipids. After their synthesis on endoplasmic reticulum (ER) membranes, SREBPs form tight complexes with SCAP. If cells are replete with sterols, the SCAP/SREBP complex binds to one of the two ER retention proteins, Insig-1 or Insig-2, which hold the SCAP/SREBP complex in the ER. When sterols are depleted, the SCAP/SREBP complex dissociates from Insigs and becomes incorporated into COPII-coated vesicles that transport the complex to the Golgi apparatus (3, 4). Here, the SREBP is processed sequentially by two proteases that release its active transcription factor activating genes required for cholesterol synthesis and other lipids. After their synthesis on ER membranes, SREBPs form tight complexes with SCAP. If cells are replete with sterols, the SCAP/SREBP complex binds to one of the two ER retention proteins, Insig-1 or Insig-2, which hold the SCAP/SREBP complex in the ER. When sterols are depleted, the SCAP/SREBP complex dissociates from Insigs and becomes incorporated into COPII-coated vesicles that transport the complex to the Golgi apparatus (3, 4). Here, the SREBP is processed sequentially by two proteases that release its active transcription factor activating genes required for cholesterol synthesis and other lipids.

Materials and Methods

Plasmids. The following recombinant expression plasmids have been described: pTK-HSV-BP-2, encoding wild-type herpes simplex virus (HSV)-tagged human SREBP-2 under control of thymidine kinase (TK) promoter (16); pCMV-SCAP, encoding wild-type hamster SCAP under control of cytomegalovirus (CMV) promoter (17); pTK-SCAP and pTK-SCAP(Y298C), encoding wild-type and mutant hamster SCAP, respectively, under control of TK promoter (18); and pTK-Insig-1-Myc and pTK-Insig-2-Myc, encoding wild-type human Insig-1 and Insig-2, respectively, followed by six tandem copies of a c-Myc epitope tag (EQKLISEEDL) under control of TK promoter (Y. Gong, M.S.B., and J.L.G., unpublished work). Site-directed mutagenesis was carried out by using the QuiKChange II XL kit (Stratagene). The coding regions of all plasmids were sequenced before use.

Cell Culture. Cells were grown in monolayer at 37°C in an atmosphere of 8–9% CO₂, SRD-13A cells are previously described to synthesize cholesterol and unsaturated fatty acid auxotrophs derived from γ-irradiated CHO cells (19) and maintained in medium A (a 1:1 ratio mixture of Ham’s F-12 medium and DMEM containing 5% FCS, 5 μg/ml cholesterol, 1 mM sodium selenomethionine, 20 μM sodium selenite, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate). Lipid auxotrophy results from a loss of the mRNA encoding SCAP.

Abbreviations: CMV, cytomegalovirus; ER, endoplasmic reticulum; 25-HC, 25-hydroxycholesterol; HCD, hydroxypropyl-β-cyclodextrin; HSV, herpes simplex virus; MCD, methyl-β-cyclodextrin; SREBP, sterol regulatory element-binding protein; TK, thymidine kinase; SCAP, SREBP cleavage-activating protein.

*To whom correspondence may be addressed. E-mail: mike.brown@utsouthwestern.edu or joe.goldstein@utsouthwestern.edu.

© 2005 by The National Academy of Sciences of the USA.
Transient Transfection of SRD-13A Cells. On day 0, SRD-13A cells were set up in medium A at 6.5 x 10^5 cells per 60-mm dish. On day 1, cells were transfected with plasmids with FuGENE 6 reagent (19). The total amount of DNA in each transfection was adjusted to 3–5 μg per dish with pcDNA3 (Invitrogen). After transfection, the cells were incubated at 37°C for 16 h. On day 2, cells were washed once with PBS, switched to medium B (a 1:1 ratio mixture of Ham's F-12 medium and DMEM containing 5% newborn calf lipoprotein-deficient serum, 50 μM sodium compactin, 50 μM sodium mevalonate, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate) containing 1% (wt/vol) hydroxypropyl-β-cyclodextrin (HCD). After incubation at 37°C for 1 h, cells were washed twice with PBS and switched to medium B in the absence or presence of sterols as indicated. After incubation for 3–6 h, cells were harvested for SREBP-2 cleavage or immunoprecipitation. Transfection of SRD-13A cells in 100-mm dishes was performed as above except that cells were set up at 7 x 10^5 cells per 100-mm dish and transfected on day 2. On day 3, cells were treated as indicated in the figure legends and harvested for blue native PAGE and trypsin cleavage assay.

Trypsin Cleavage of SCAP. Aliquots (100 μg) of the 20,000 g membrane fraction from transfected SRD-13A cells were subjected to trypsin digestion, followed by electrophoresis on 12% Tris-tricine gels as described (15).

Purification of Recombinant SCAP. Recombinant baculoviruses encoding the eight transmembrane regions of wild-type or D428A mutant version of hamster SCAP with an NH2-terminal His tag, designated His10-SCAP(TM1–8) or His10-SCAP(TM1–8)(D428A), were constructed in pFastBacHTa expression vector (Invitrogen) as described (12). Recombinant proteins were...
overexpressed in SF9 insect cells and purified in buffer D [50 mM Tris-HCl, pH 7.5/150 mM NaCl/1 mM DTT/0.1% (wt/vol) Fos-Choline] by using nickel chromatography and gel filtration as described (12).

**[3H]Sterol-Binding Assay.** In the standard assay, binding reactions were set up in microcentrifuge tubes at room temperature as described (12). Each reaction, in a final volume of 100 μl of buffer D contained 10 pmol (0.8 μg) of purified His10-SCAP(TM1–8) or His10-SCAP(TM1–8)(D428A), 1–40 pmol of [3H]cholesterol or [3H]25-HC solubilized in buffer D (10–400 nM, final concentration), and 25 mM phosphorylcholine chloride. After incubation for 4 h, the mixture was passed through a column packed with 0.3 ml of Ni-nitrolotriacetic acid (NTA) agarose beads. Each column was then washed for ~20 min with 10 ml of buffer D, after which protein-bound [3H]cholesterol was eluted with 250 mM imidazole as described (12). Dissociation rates were measured by isolating the [3H]cholesterol/SCAP complex as described above, and diluting it 10-fold with buffer D saturated with unlabeled cholesterol. After incubation at room temperature for the indicated time, the mixture was transferred to a tube containing 1 ml of Ni-NTA agarose beads pre-equilibrated with buffer D, incubated for 2 min, and then centrifuged at 400 × g for 30 s, after which the pellet and supernatant were assayed for radioactivity by scintillation counting (12).

Further information on materials and certain methods used in this study (SREBP-2 Cleavage Assay, Immunoprecipitation, Immunoblot Analysis, Blue Native-PAGE, and Solubilization of Sterols) can be found in Supporting Methods, which is published as supporting information on the PNAS web site.

**Results**

Fig. 4A shows the amino acid sequence and proposed topology of the membrane domain of hamster SCAP. Residues that are identical in SCAP sequences from seven animal species are indicated in red. Residues that are also identical in bread mold are indicated in green. In an initial attempt to identify functionally important residues, we prepared plasmids encoding mutant SCAPs in which the conserved residues were changed individually. To test the significance of these individually important residues, we prepared plasmids encoding mutant SCAPs, which is published (12). Dissociation rates were measured by isolating the [3H]cholesterol/SCAP complex as described above, and diluting it 10-fold with buffer D saturated with unlabeled cholesterol. After incubation at room temperature for the indicated time, the mixture was transferred to a tube containing 1 ml of Ni-NTA agarose beads pre-equilibrated with buffer D, incubated for 2 min, and then centrifuged at 400 × g for 30 s, after which the pellet and supernatant were assayed for radioactivity by scintillation counting (12).

Fig. 4A shows the amino acid sequence and proposed topology of the membrane domain of hamster SCAP. Residues that are identical in SCAP sequences from seven animal species are indicated in red. Residues that are also identical in bread mold are indicated in green. In an initial attempt to identify functionally important residues, we prepared plasmids encoding mutant SCAPs in which the conserved residues were changed individually to alanine. The plasmids were introduced into SRD-13A wild-type SCAP, or two mutant SCAPs, were transfected into SRD-13A cells together with a plasmid encoding SREBP-2. When SREBP-2 was overexpressed by transfection, the full-length protein was visualized in membranes, but it was not processed to the nuclear form (Fig. 2, lane 2). Coexpression of
wild-type SCAP at low levels (0.2–0.5 μg of plasmid) restored SREBP-2 processing (Fig. 2, lanes 3 and 5), and this expression was decreased by 25-HC (Fig. 2, lanes 4 and 6). At a higher level of SCAP expression, there was no longer suppression by 25-HC (Fig. 2, lanes 7 and 8), owing to the excess of SCAP over endogenous Insig as previously reported (1). Transfection with 0.5 μg of the SCAP(D248A) plasmid failed to produce SREBP-2 processing (Fig. 2, lanes 9 and 10), despite the fact that the amount of SCAP expression was comparable with that observed with 0.5 μg of wild-type SCAP (see immunoblot of SCAP in membranes; Fig. 2, lanes 5–12). At higher levels of expression, the D248A mutant was able to support some SREBP-2 processing (Fig. 2, lanes 11 and 13), but the amount was much less than observed at similar levels of wild-type SCAP (Fig. 2, lanes 7 and 8).

For comparative purposes in this experiment, we transfected the SRD-13A cells with a plasmid encoding the Y298C mutant of SCAP, which does not bind to Insig, and therefore does not undergo inhibition by 25-HC (1). SCAP(Y298C) stimulated SREBP-2 processing, and there was no inhibition by 25-HC (Fig. 2, lanes 15–20). To quantify these data, the gels were scanned on a densitometer, and the intensity of the nuclear SREBP-2 band was plotted as a function of the density of the SCAP band (Fig. 2B). The data show that wild-type SCAP and SCAP(Y298C) were roughly equivalent in their ability to support SREBP-2 processing, but SCAP(D248A) was markedly deficient.

For SCAP to carry SREBPs to the Golgi, SCAP must dissociate from Insig proteins in a reaction that requires sterol depletion. The experiments of Fig. 3 were designed to determine whether SCAP(D248A) binds to Insig, and whether it dissociates in the absence of sterols. We previously showed that the SCAP/Insig complex can be visualized as a slow-moving series of broad bands when cell extracts are processed by blue native PAGE and blotted with an antibody to epitope-tagged Insig-1 (1, 15). When Myc-tagged Insig-1 was expressed alone in SRD-13A cells, no complex with SCAP was observed (Fig. 3A, lanes 1 and 2). Coexpression with wild-type SCAP produced a SCAP/Insig complex that was observed in the presence of sterols (Fig. 3A, lane 4), but disappeared upon sterol depletion (Fig. 3A, lane 3). SCAP(Y298C) did not form a complex with Insig under either condition (Fig. 3A, lanes 5 and 6). In marked contrast, SCAP(D248A) formed a complex with Insig in the absence or presence of sterols (Fig. 3A, lane 7). It did not pull down SCAP(Y298C) in either condition (Fig. 3B, lanes 4 and 5), and it pulled down
SCAP(D428A) under both conditions (Fig. 3B, lanes 6 and 7). These data indicate that the D428A substitution alters SCAP in such a way that it binds to Insig constitutively in the absence and presence of sterols.

When SCAP binds to cholesterol and Insigs, it undergoes a conformational change that exposes a membrane-proximal arginine residue to protease cleavage (13–15). This change produces a lower band that is visualized when sealed vesicles are treated with trypsin, subjected to SDS/PAGE, and blotted with an antibody against a protected intraluminal loop of SCAP. As shown in Fig. 4A, when sterol-depleted cells expressed wild-type SCAP trypsin digestion produced a predominant upper band (Fig. 4A, lane 1). Preincubation of the membrane vesicles with increasing amounts of cholesterol/cyclodextrin complexes led to a gradual increase in the amount of the lower band (Fig. 4A, lanes 2–5). 25-HC/cyclodextrin treatment did not produce the lower band (Fig. 4A, lanes 6 and 7). When cells expressed SCAP(D428A), the lower band was visualized even in the absence of sterols (Fig. 4A, lane 1), and it reached a near maximum at the lowest cholesterol concentration (10 μM). To quantify these results, the gels were scanned, and the density of the lower band was expressed as a percentage of the total density of both bands (Fig. 4C). In the absence of added cholesterol, >50% of SCAP(D428A) was present in the lower band, and nearly 100% was in this form at 10 μM of cholesterol. This result was markedly different from the result with wild-type SCAP.

The conformational change in SCAP can also be elicited by treatment of the cells with certain amphipathic amines like chlorpromazine (14). SCAP(D428A) shared the properties of wild-type SCAP, we treated the cells with certain amphipathic amines like chlorpromazine (14). SCAP(D428A) also hypersensitive to the treatment of the cells with certain amphipathic amines like chlorpromazine (14). SCAP(D428A) under both conditions (Fig. 3A). Thus, the membrane domain of SCAP(D428A) was indistinguishable in biochemical properties from its wild-type counterpart.

To determine whether the defect in SCAP(428A) is due to the loss of the negative charge, we prepared a series of plasmids encoding SCAP with various substitutions at this position and transfected them into SRD-13A cells (Fig. 6A). Substitution of glutamic acid for aspartic acid preserved most of the ability of SCAP to facilitate SREBP-2 cleavage in the absence of sterols (Fig. 6A, lane 6). Substitution with alanine, asparagine, or lysine destroyed this function (Fig. 6A, lanes 4, 8, and 10). In addition, we coimmunoprecipitated SCAP(D428A) with Insig-2 equally in the absence and presence of sterols, except for the D428E mutant, which consistently showed an increased dissociation from Insig-2 in the absence of sterols (Fig. 6B, lane 6). In the trypsin cleavage assay, the D428E mutant showed only a small amount of lower band in the absence of cholesterol, and this amount increased in a normal fashion as the cholesterol concentration increased (Fig. 6C). Considered together, these data suggest that SCAP requires a negatively charged amino acid at position 428 to dissociate from Insigs in the absence of cholesterol. Aspartic acid functions somewhat better than does glutamic acid in this regard.

### Discussion

The current studies disclose a functional role for the universally conserved negatively charged aspartic acid near the middle of the sixth transmembrane helix of SCAP. When Asp–428 was changed to alanine, SCAP bound more tightly to Insigs in sterol-depleted cells, and the mutant protein was no longer able to escort SREBP-2 efficiently to the Golgi. The change in SCAP’s behavior was not caused by denaturation or unfolding, as revealed by the normal physical properties of the purified membrane domain of the mutant protein (Fig. 5). The increased binding of SCAP(D428A) to Insigs in sterol-depleted cells was correlated with an increase in the proportion of SCAP that was in the Insig-binding conformation, as indicated by the abundant lower band on trypsin digestion of isolated
membranes (Fig. 4). The proportion of SCAP in this conformation increased in a hypersensitive fashion when SCAP-containing membranes were incubated with low concentrations of cholesterol or amphiphatic amines (Fig. 4). These data raise the possibility that SCAP(D428A) may bind to Insigs in sterol-depleted cells because it is hypersensitive to cholesterol, and, therefore, it remains in its Insig-binding conformation, owing to small amounts of cholesterol that may remain in ER membranes even when cells have been treated with HCD.

Although SCAP(D428A) was hypersensitive to cholesterol when embedded in membranes containing Insigs, the mutant protein did not show an increased affinity for cholesterol binding when studied in solution in the absence of Insigs (Fig. 5). Protein did not show an increased affinity for cholesterol binding when embedded in membranes containing Insigs, the mutant even when cells have been treated with HCD.

It is likely that Asp-428 may play other roles in SCAP function, in addition to accelerating Insig binding. This conclusion follows from the observation that Asp-428 is conserved in SCAP from Drosophila, which do not have a recognizable Insig gene. In Drosophila cells, SCAP movement is not inhibited by sterols; rather, it is inhibited by phosphatidyl ethanolamine (20). Studies in insect cells should reveal whether Asp-428 plays a role in this process.

An important aspect of the current studies is that they provide genetic evidence that our in vitro assays in mammalian cells reflect functionally relevant changes in SCAP. Thus, the D428A mutation increases the proportion of SCAP in the Insig-binding conformation, as determined by the in vitro trypsin cleavage assay, increases the binding of SCAP to Insigs as determined by in vitro blue native PAGE or immunoprecipitation, and decreases the proteolytic processing of SREBP-2 in intact cells. This correlation between in vitro assays and biological effects in intact cells supports the notion that SCAP's conformation is the central determinant that dictates the rate of SREBP processing, and therefore, the rate of lipid synthesis in mammalian cells.

We thank Jin Ye and Chris Adams for helpful discussion, Angela Carroll and Lisa Beatty for assistance with tissue culture, and Jeff Cormier for DNA sequencing. This work was supported by National Institutes of Health Grant HL09048, the Perot Family Foundation, and National Institutes of Health Medical Scientists Training Grant GM08014 (to J.D.F.). A.R. is a Fellow of the Jane Coffin Childs Memorial Fund for Medical Research. Y.I. is a Fellow of the Banyu Life Science Foundation International.