Transport-Dependent Proteolysis of SREBP: Relocation of Site-1 Protease from Golgi to ER Obviates the Need for SREBP Transport to Golgi

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Summary

Cholesterol homeostasis in animal cells is achieved by regulated cleavage of membrane-bound transcription factors, designated SREBPs. Proteolytic release of the active domains of SREBPs from membranes requires a sterol-sensing protein, SCAP, which forms a complex with SREBPs. In sterol-depleted cells, SCAP escorts SREBPs from ER to Golgi, where SREBPs are cleaved by Site-1 protease (S1P). Sterols block this transport and abolish cleavage. Relocating active S1P from Golgi to ER by treating cells with brefeldin A or by fusing the ER retention signal KDEL to S1P obviates the SCAP requirement and renders cleavage insensitive to sterols. Transport-dependent proteolysis may be a common mechanism to regulate the processing of membrane proteins.

Introduction

Cleavage of sterol regulatory element binding proteins (SREBPs) by Site-1 protease (S1P) initiates a process by which the active fragments of the SREBPs translocate to the nucleus and activate genes controlling the synthesis and uptake of cholesterol and unsaturated fatty acids in animal cells (Brown and Goldstein, 1999). The SREBPs are synthesized as tripartite membrane-bound proteins averaging 1150 amino acids in length. The NH$_2$-terminal segment of ~480 amino acids is a transcription factor of the basic helix-loop-helix-leucine zipper (bHLH-Zip) family. This is followed by an 80–amino acid membrane attachment domain that comprises two transmembrane helices separated by a short hydrophilic loop of ~30 amino acids. This is followed by a COOH-terminal regulatory domain of ~590 amino acids. Newly synthesized SREBPs are bound to membranes of the nuclear envelope and endoplasmic reticulum (ER) in a hairpin fashion with the NH$_2$-terminal and COOH-terminal segments projecting into the cytosol and the hydrophilic loop projecting into the lumen (Brown and Goldstein, 1997).

The newly synthesized SREBPs form complexes with a polytopic membrane protein designated SCAP (SREBP cleavage-activating protein) (Sakai et al., 1997, 1998a). The NH$_2$-terminal domain of SCAP contains eight transmembrane helices, and the COOH-terminal domain contains five WD40 repeats that mediate protein–protein interactions (Nohturfft et al., 1998a). The SREBP/SCAP

complex is formed by an interaction between the cytosolic COOH-terminal regulatory domain of the SREBPs and the cytosolic WD40 domain of SCAP. SCAP targets the SREBPs to S1P, a membrane-bound serine protease whose active site faces the lumen of the ER and post-ER organelles (Sakai et al., 1998b). S1P cleaves the luminal loop of the SREBPs following the tetrapeptide sequence Arg-Xaa-Xaa-Leu, thereby separating SREBP into two halves (Duncan et al., 1997). This event allows a second protease, designated Site-2 protease (S2P), to cleave the NH$_2$-terminal transmembrane domain, thereby liberating the NH$_2$-terminal segments of SREBPs from the membrane and allowing them to enter the nucleus (Sakai et al., 1996; Rawson et al., 1997).

This complex proteolytic activation mechanism allows lipid synthesis to be controlled by the lipid content of cell membranes (Brown and Goldstein, 1997). When cells are depleted of sterols, the cleavage reactions are rapid, and the NH$_2$-terminal segments of SREBPs readily enter the nucleus. When cells are overloaded with sterols, the Site-1 cleavage reaction is blocked. The SREBPs remain bound to cell membranes, and the target genes are no longer activated. Cholesterol synthesis stops as a result of decreased transcription of genes encoding 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase, HMG CoA reductase, and other enzymes of the cholesterol biosynthetic pathway. Cholesterol uptake is blocked by a downregulation of the low-density lipoprotein (LDL) receptor, and the synthesis of unsaturated fatty acids is partially decreased as a result of reduced transcription of genes encoding acetyl CoA carboxylase, fatty acid synthase, and stearoyl CoA desaturase (Brown and Goldstein, 1997; Edwards and Ericsson, 1999).

Biochemical and genetic data show that SCAP is the key to the regulation of Site-1 cleavage (Hua et al., 1996a; Nohturfft et al., 1996, 1999; Rawson et al., 1999). Mutant Chinese hamster ovary (CHO) cells that lack SCAP fail to cleave SREBPs at Site-1, and they are therefore auxotrophic for cholesterol and unsaturated fatty acids (Rawson et al., 1999). Sterols block Site-1 cleavage by reducing the activity of SCAP. This inhibition does not occur when SCAP bears substitution mutations at either of two positions in its membrane attachment domain (Tyr298Cys or Asp443Asn) (Hua et al., 1996a; Nohturfft et al., 1996, 1998b). This portion of the membrane attachment domain has been designated the sterol-sensing region. Mutant CHO cells that produce these altered forms of SCAP overproduce cholesterol and fail to slow this synthesis when cholesterol overaccumulates in the cell.

Evidence indicates that SCAP acts by escorting SREBPs to a post-ER compartment where the active S1P resides and that sterols may prevent cleavage by blocking this transport (Nohturfft et al., 1998b, 1999). This hypothesis emerged from studies of the N-linked carbohydrates attached to a luminal loop in the polytopic membrane domain of SCAP. When cells are depleted of sterols, these carbohydrates are largely resistant to cleavage by endoglycosidase H (endo H),

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indicating that SCAP has reached the Golgi apparatus where it has been modified by Golgi mannosidase II (Kornfeld and Kornfeld, 1985). When cells are over-loaded with sterols, the N-linked sugars of SCAP remain in an endo H–sensitive form, indicating that SCAP has not reached the Golgi. In sterol-depleted cells, most of the SCAP cofractionates with ER markers even though it has been modified by Golgi enzymes (Nohturfft et al., 1999). These findings have led to a model in which SCAP carries SREBP from the ER to the Golgi in sterol-depleted cells, after which SCAP recycles to the ER following Site-1 cleavage. In sterol-overloaded cells, the SCAP/SREBP complex does not leave the ER and Site-1 cleavage cannot take place.

A corollary of the sterol-regulated cycling model is that S1P is not active in the ER and that it becomes activated only after transport to a post-ER compart-ment. Partial support for this model comes from experi-ments on the activation process of S1P. S1P, like other mammalian subtilases, is synthesized in the ER as an inactive precursor (Espenshade et al., 1999). It becomes active only after it is autocatalytically cleaved, liberating an NH_2-terminal propeptide. Most of the inactive S1P precursor, designated S1P-A, is endo H sensitive, sug-gesting that the protein is largely located in the ER. On the other hand, the major active form of S1P, designated S1P-C, is endo H resistant, suggesting that it resides in the Golgi (Espenshade et al., 1999).

If the primary function of SCAP is to transport SREBP to the post-ER compartment that contains active S1P, the requirement for SCAP should be abolished if we could generate active S1P in the ER. The current experi-ments were designed to test this hypothesis using mu-tant SRD-13A cells that lack SCAP (Rawson et al., 1999). We used two approaches to trap active S1P in the ER. First, we treated the SRD-13A cells with brefeldin A, an agent that blocks anterograde movement of proteins from ER to Golgi, thus causing Golgi proteins to translo-cate back to the ER (Lippincott-Schwartz et al., 1989). Second, we transfected the SRD-13A cells with a cDNA encoding a truncated, soluble luminal form of S1P with an ER retention signal inserted at its COOH terminus. Both of these treatments led to Site-1 cleavage of SREBPs in the SCAP-deficient SRD-13A cells, and in both cases this SCAP-independent cleavage was not inhibited by sterols. These data provide strong experi-mental support for the sterol-regulated transport model of SCAP function.

Results

Figure 1 shows an experiment designed to test the ef-fects of brefeldin A on sterol-regulated cleavage of SREBP-2 in wild-type CHO-7 cells. In panel A, the cells were incubated for 16 hr without sterols to induce cleav-age of SREBPs. The cells were then incubated for an additional 5 hr in the absence or presence of a mixture of 25-hydroxycholesterol and cholesterol, which efficiently suppresses Site-1 cleavage. Extracts of cell membranes and nuclei were then subjected to SDS-PAGE and blot-ted with an antibody against the NH_2-terminal segment of SREBP-2. As expected, the NH_2-terminal segment was found in the nuclear extract when the cells were
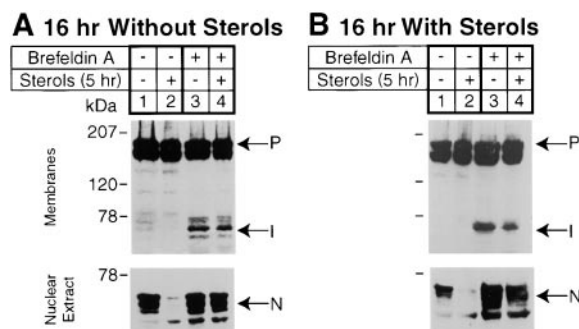

Figure 1. Brefeldin A Blocks Sterol-Mediated Suppression of SREBP Cleavage in CHO Cells

On day 0, CHO-7 cells were set up at 5×10^5 cells per 10 cm dish in medium A supplemented with 5% fetal calf serum. On day 2, the cells were switched to medium A supplemented with 5% newborn calf lipoprotein-deficient serum and compactin/mevalonate in the absence (A) or presence (B) of sterols. After 16 hr, the cells were switched to medium A supplemented with 5% newborn calf lipopro-tein-deficient serum and compactin/mevalonate in the absence or presence of sterols and 1 μg/ml brefeldin A as indicated. After 5 hr, the cells were harvested, and membrane and nuclear extract fractions were prepared as described in the Experimental Proce-dures. Aliquots of the membranes (50 μg protein) and nuclear ex-tracts (100 μg protein) were subjected to SDS-PAGE. Immunoblot analysis was performed with 5 μg/ml of monoclonal IgG-7D4, which recognizes SREBP-2. The filters were exposed to Kodak X-Omat Blue XB-1 film for 10 s (membranes) and 1 min (nuclear extracts) at room temperature. P, I, and N denote the precursor, intermediate, and nuclear forms of SREBP, respectively.

incubated in the absence of sterols (lower panel, lane 1) but not in the presence of sterols (lane 2). When the final 5 hr incubation was conducted in the presence of brefeldin A, nuclear SREBP-2 continued to be visualized in the sterol-depleted cells (lane 3), but there was no longer any decrease when sterols were added (lane 4). The amount of the membrane-bound precursor did not change under any of these conditions (upper panel). Brefeldin A did produce an increase in the membrane-bound intermediate of SREBP (designated I in Figure 1), which is the immediate product of Site-1 cleavage. This experiment shows that brefeldin A blocks the sup-pression of Site-1 cleavage by sterols.

Panel B of Figure 1 shows that brefeldin A induces SREBP cleavage in cells that were previously inhibited by sterols. In this experiment, the cells were incubated for 16 hr in the presence of sterols, and then some of the dishes were switched to sterol-free medium in the absence or presence of brefeldin A. In the absence of brefeldin A, we found no nuclear SREBP-2 in the sterol-treated cells (panel B, lane 2). In the presence of brefel-din A, nuclear SREBP-2 appeared even when sterols were present (lane 4). Again, brefeldin A blocked sterol suppression of SREBP cleavage.

A trivial explanation for the findings in Figure 1 would exist if brefeldin A blocked the movement of the exoge-nous sterols to the ER. This possibility is rendered un-likely by the prior findings of Ridgway and Lagace (1995), who showed that brefeldin A does not interfere with the ability of 25-hydroxycholesterol to stimulate the ER synthesis of cholesteryl esters in CHO cells. This result was confirmed in our laboratory (Nohturfft et al., 1999).
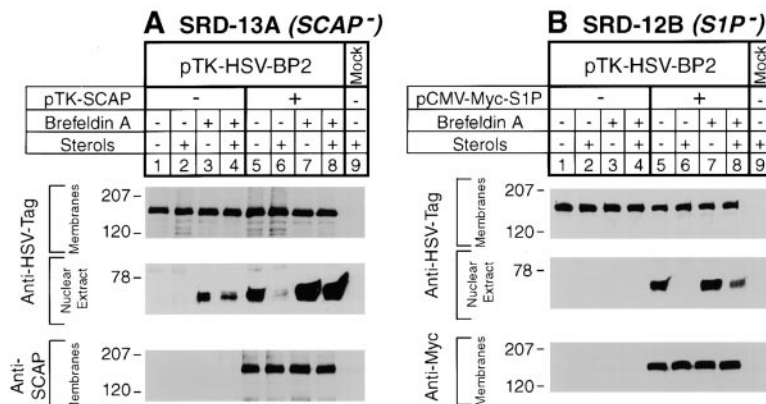
A SRD-13A *(SCAP⁻)*

pTK-HSV-BP2									Mock
pTK-SCAP	-				+				
Brefeldin A	-	-	+	+	-	-	+	+	-
Sterols	-	+	-	+	-	+	-	+	+
	1	2	3	4	5	6	7	8	9

B SRD-12B *(S1P⁻)*

pTK-HSV-BP2									Mock
pCMV-Myc-S1P	-				+				
Brefeldin A	-	-	+	+	-	-	+	+	-
Sterols	-	+	-	+	-	+	-	+	+
	1	2	3	4	5	6	7	8	9

Figure 2. Brefeldin A Induces S1P-Dependent Cleavage of SREBPs in Transfected Cells

(A) On day 0, SCAP-deficient SRD-13A cells were set up at a density of 4×10^5 cells/60 mm dish in medium B. On day 1, cells were transfected with 2 μg/dish of pTK-HSV-BP2 together with 0.25 μg/dish pTK-SCAP. The total amount of DNA was adjusted to 4 μg/dish with pcDNA3 empty vector and pTK mock vector. On day 2, the cells were cultured for 16 hr in medium A supplemented with 5% newborn calf lipoprotein-deficient serum and compactin/mevalonate in the absence or presence of sterols. On day 3, cells were incubated with or without 1 μg/ml brefeldin A for 5 hr, after which they were harvested and fractionated as described in Figure 1. Aliquots of membranes (6 μg protein) and nuclear extracts (10 μg protein) were subjected to SDS-PAGE, and immunoblot analysis was carried out with 0.1 μg/ml of IgG-HSV-Tag for pTK-HSV-BP2 and 5 μg/ml IgG-R139 for SCAP. Filters were exposed to film for 1 s (top and bottom panels) and 30 s (middle panel). Molecular mass standards are expressed in kilodaltons.
(B) On day 0, S1P-deficient SRD-12B cells were set up at a density of 3×10^5 cells/60 mm dish in medium B. On day 1, cells were transfected with 2 μg/dish of pTK-HSV-BP2 together with 0.5 μg/dish pCMV-Myc-S1P. The total amount of DNA was adjusted to 4 μg/dish with pcDNA3 empty vector and pTK mock vector. On day 2, the cells were cultured for 16 hr in the absence or presence of sterols as described in (A). On day 3, cells were treated with 1 μg/ml brefeldin A for 5 hr prior to harvest and fractionation. Aliquots of membranes (14 μg protein) and nuclear extract (33 μg protein) were subjected to SDS-PAGE, and immunoblot analysis was carried out with 0.1 μg/ml of IgG-HSV-Tag for pTK-HSV-BP2 and 0.5 μg/ml anti-Myc 9E10 for Myc-S1P. Filters were exposed to film for 5 s (top panel), 15 s (middle panel), and 1 s (bottom panel). Molecular mass standards are expressed in kilodaltons.

If the effect of brefeldin A on SREBP cleavage is caused by relocation of active S1P to the ER, then brefeldin A should relieve the requirement for SCAP. To test this hypothesis, we measured the cleavage of epitope-tagged SREBP-2 in transfected SRD-13A cells, which lack SCAP as a result of mutations in the *SCAP* gene (Figure 2A). In the absence of SCAP and brefeldin A, these cells showed no nuclear SREBP-2 whether incubated in the absence or presence of sterols (middle panel, lanes 1 and 2). Treatment with brefeldin A caused the appearance of nuclear SREBP-2, and this was not prevented by sterols (lanes 3 and 4). As a positive control for these experiments, we transfected the cells with a cDNA encoding wild-type SCAP (lanes 5–8). In the absence of brefeldin A, the *SCAP* cDNA restored nuclear SREBP-2, and cleavage was blocked by sterols (lanes 5 and 6). Brefeldin A allowed cleavage to persist in the presence of sterols (lanes 7 and 8). To confirm that the nuclear SREBP-2 resulted from cleavage by Site-1 protease, we performed a similar experiment in SRD-12B cells, which lack S1P as a result of mutations in the *S1P* gene (Figure 2B). In the absence of S1P, brefeldin A failed to cause the appearance of nuclear SREBP-2 either in the absence or presence of sterols (middle panel, lanes 1–4). When we cotransfected a cDNA encoding S1P, we restored cleavage of SREBP-2, and this was inhibited by sterols (lanes 5 and 6). Sterol suppression was blocked by brefeldin A (lanes 7 and 8). Thus, treatment of SCAP-deficient cells with brefeldin A rescues S1P-dependent cleavage of SREBPs.

To study the subcellular localization of S1P directly, we performed double-label immunofluorescence studies of CHO/pS2P cells and S1P-deficient SRD-12B cells, which were derived from the CHO/pS2P cells (Figure 3). When stained with an antibody against S1P, the CHO/pS2P cells exhibited intense staining of a juxtanuclear organelle whose appearance was consistent with that of the Golgi complex (Figure 3A). This intense staining was absent in the SRD-12B cells (Figure 3D), indicating that it was specific for S1P. We also observed a faint reticular staining pattern that appeared nonspecific since it was present in the SRD-12B cells (Figure 3D) as well as the CHO/pS2P cells (Figure 3A). Because of this nonspecific staining, we could not determine whether any S1P is located in the ER. Incubation of cells with rhodamine-conjugated wheat germ agglutinin (WGA) stained the *trans*-Golgi network in the CHO/pS2P cells (Figure 3B) and the SRD-12B cells (Figure 3E). Overlay of the green S1P image with the red WGA image revealed that S1P and the WGA Golgi marker colocalized in CHO/pS2P cells, as indicated by the merged yellow image (Figure 3C). No colocalization was observed in the SRD-12B cells, which is consistent with the absence of S1P in these cells (Figure 3F).

We used the same technique to trace the fate of S1P in brefeldin A–treated CHO/pS2P cells (Figure 4). In the absence of brefeldin A, the anti-S1P antibody showed intense Golgi fluorescence and a small amount of reticular fluorescence (Figure 4A). When the cells were treated with brefeldin A, the Golgi fluorescence disappeared, and the reticular pattern increased (Figure 4D). Staining with WGA revealed a Golgi pattern (Figure 4B), and this was unaffected by brefeldin A (Figure 4E). This finding is consistent with previous data showing that brefeldin A causes the *cis*- and medial-Golgi to collapse into the ER, but it does not affect the *trans*-Golgi (Lippincott-Schwartz et al., 1989). Overlay of the S1P and WGA images confirmed the Golgi localization of S1P in the untreated cells (Figure 4C), and this was abolished in the brefeldin A–treated cells (Figure 4F). This finding suggests that S1P is located in the *cis*- and medial-Golgi, but not in the *trans*-Golgi.

We previously reported that S1P is processed to three forms, designated S1P-A, S1P-B, and S1P-C (Espenshade et al., 1999). The NH$_2$-terminal sequences of these
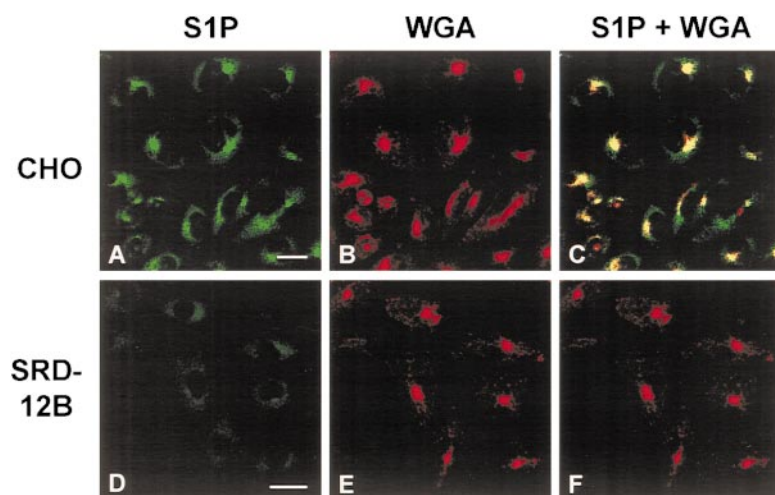
Figure 3. Endogenous S1P Localizes to the Golgi Apparatus in CHO Cells

Wild-type CHO/pS2P cells (A–C) or S1P-deficient SRD-12B cells (D–F) were grown in medium B, fixed, and stained with antibodies to S1P (A and D) or rhodamine-conjugated wheat germ agglutinin (B and E) as described in the Experimental Procedures. Merged images (C and F) show colocalization of S1P and the Golgi marker, wheat germ agglutinin. Bar, 20 μm.

proteins were examined, and this revealed that S1P-A is the product of cleavage by signal peptidase. S1P-A retains the propeptide, and it is enzymatically inactive. S1P-B and S1P-C are produced by autocatalytic removal of the propeptide, and they are enzymatically active. The N-linked carbohydrates on S1P-A and S1P-B are mostly endo H sensitive, indicating that the bulk of these proteins have not reached the medial-Golgi. S1P-C is endo H resistant, implying that it has reached the medial-Golgi. All of these experiments were performed with transfected cells that overexpressed epitope-tagged S1P. To confirm that these same findings apply to endogenous S1P, we performed immunoblotting experiments with anti-S1P in nontransfected CHO cells (Figure 5). As reported previously, we observed S1P-A, S1P-B, and S1P- C in SRD-12B cells that overexpress transfected S1P (Figure 5A, lane 2). In contrast, the nontransfected CHO cells showed only two bands, corresponding in size to S1P-A and S1P-C (Figure 5A, lane 3). This indicates that S1P-C is the major form of active S1P in nontransfected cells and that S1P-B becomes prominent only when the protein is overexpressed as a result of transfection. When CHO cell membranes were treated with peptide N-glycosidase F

(PNGase F), the mobility of the A and C forms increased, indicating that both forms have N-linked sugars (Figure 5B, lanes 2 and 3). Endo H treatment increased the mobility of S1P-A to approximately the same extent as PNGase F, indicating that all of the N-linked sugars were endo H sensitive (Figure 5B, lane 4). The small difference in mobility between PNGase F–treated and endo H–treated S1P-A may be due to the fact that endo H does not remove the asparagine-linked N-acetylglucosamine whereas PNGase F does. The mobility of S1P-C was only partially increased by endo H, and none of it comigrated with the PNGase F product. These data indicate that all molecules of S1P-C contain at least one endo H–resistant carbohydrate chain, implying that this protein has reached the medial-Golgi. Similar results were obtained when the cells were incubated with sterols (Figure 5B, lanes 7–10), which is consistent with previous evidence that sterols do not affect the autocatalytic processing or subcellular localization of S1P (Espenshade et al., 1999). Together, these data suggest that in nontransfected cells the active form of S1P, namely S1P-C, resides in the Golgi.

In addition to brefeldin A treatment, another means to relocate active S1P from the Golgi to the ER is to
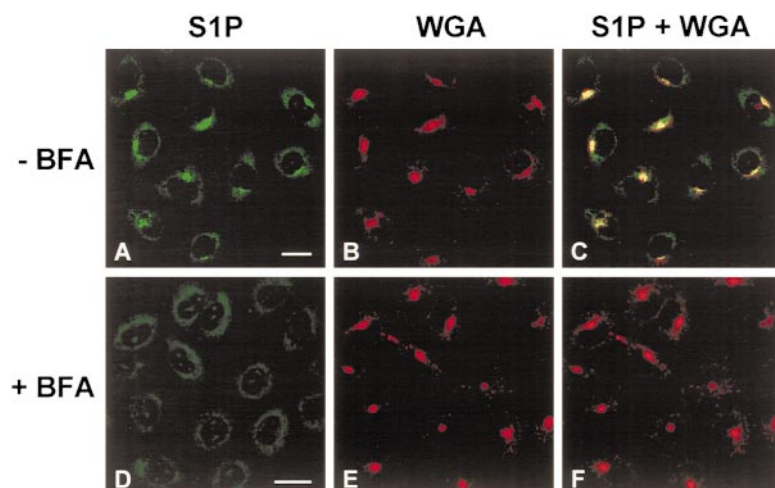

Figure 4. Brefeldin A Treatment Disrupts Golgi Localization of S1P in CHO Cells

Wild-type CHO/pS2P cells were grown in medium B and cultured for 1 hr in the absence (A–C) or presence (D–F) of 2 μg/ml brefeldin A. Cells were fixed and stained with antibodies to S1P (A and D) or rhodamine-conjugated wheat germ agglutinin (B and E) as described in the Experimental Procedures. Merged images (C and F) show localization of S1P and the Golgi marker, wheat germ agglutinin. Bar, 20 μm.
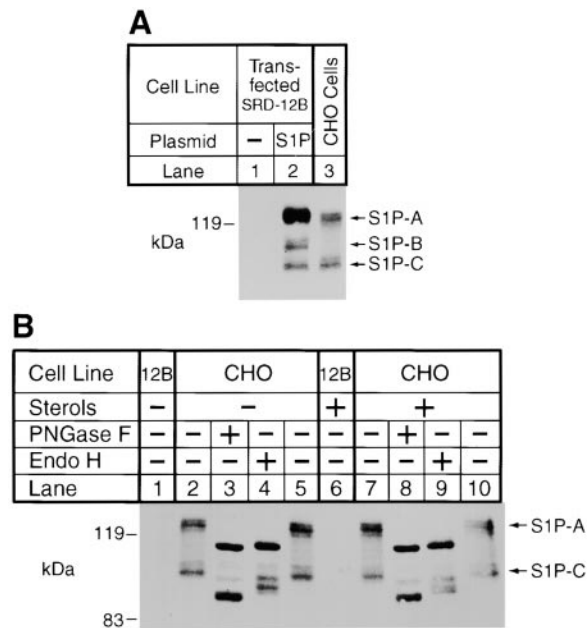
A

Cell Line	Trans-fected SRD-12B		CHO Cells
Plasmid	−	S1P	
Lane	1	2	3

119—
kDa

←S1P-A
←S1P-B
←S1P-C

B

Cell Line	12B	CHO				12B	CHO			
Sterols	−	−				+	+			
PNGase F	−	−	+	−	−	−	−	+	−	−
Endo H	−	−	−	+	−	−	−	−	+	−
Lane	1	2	3	4	5	6	7	8	9	10

119—
kDa

83—

←S1P-A
←S1P-C

Figure 5. Immunoblot Analysis of S1P in Transfected and Nontransfected CHO Cells

(A) Membrane fractions were prepared from either transfected S1P-deficient SRD-12B cells (lanes 1 and 2) or nontransfected CHO/pS2P cells (lane 3) as described in Experimental Procedures. SRD-12B cells were transfected as described in Figure 2 with either 0.5 μg of pcDNA3 empty vector (lane 1) or pCMV-S1P (lane 2), and the total amount of transfected DNA was adjusted to 3 μg/dish by the addition of pcDNA3 empty vector. Transfected cells were incubated in medium A supplemented with 5% fetal calf serum for 16 hr prior to harvest and cell fractionation. Aliquots of membrane protein (3 μg, lanes 1 and 2; 15 μg, lane 3) were subjected to SDS-PAGE and immunoblotted with a 1:500 dilution of anti-S1P antiserum. Filter was exposed to film for 10 s.
(B) CHO/pS2P and SRD-12B cells were cultured in medium A supplemented with 5% newborn calf lipoprotein-deficient serum and compactin/mevalonate in the absence (lanes 1–5) or presence of sterols (lanes 6–10) for 17 hr. Cells were harvested, and membrane fractions were prepared as described in the Experimental Procedures. Aliquots of membrane protein (15 μg) were treated with the following glycosidases: lanes 1, 2, 5, 6, 7, and 10, none; lanes 3 and 8, 0.26 IU/ml peptide N-glycosidase F (PNGase F); lanes 4 and 9, 0.83 IU/ml endo H. Following enzymatic treatment, samples were subjected to SDS-PAGE and immunoblotted with a 1:500 dilution of anti-S1P antiserum. Filter was exposed to film for 15 s.

transfect cells with a mutant form of S1P that contains an ER retention/retrieval signal. For this purpose, we prepared a cDNA encoding S1P that was truncated just prior to the COOH-terminal transmembrane domain (Figure 6A). Prior studies have shown that such a truncated form of S1P is catalytically active (Cheng et al., 1999). At the COOH terminus, we inserted three copies of a Myc epitope tag followed by the sequence Lys-Asp-Glu-Leu (KDEL). This mutant version of S1P should be inserted into the ER lumen. After it exits from the ER, it should bind to the KDEL receptor, which should transport it back to the ER from the Golgi (Pelham, 1992). As a control, we prepared a similar construct containing the COOH-terminal sequence Lys-Asp-Ala-Ser (KDAS), which is not recognized by the KDEL receptor (Munro and Pelham, 1987). We transfected these cDNAs into

SRD-12B cells, which lack endogenous S1P, and we isolated permanent clones of cells that grow in the absence of cholesterol as a result of expressing active S1P.

Figure 6B shows SREBP-2 processing in the parental SRD-12B cells and the two lines of permanent transfectants, designated S1P-KDEL and S1P-KDAS. When SRD-12B cells were incubated in the absence of sterols, the amount of SREBP-2 precursor was relatively low, and no cleaved nuclear SREBP-2 was visualized (Figure 6B, lane 1). Addition of sterols caused an increase in the amount of precursor, and there was still no nuclear SREBP-2 (lane 2). (We previously observed low amounts of endogenous SREBP precursors in sterol-deprived SRD-12B cells (Rawson et al., 1998), which we attribute to degradation of the precursor by pathways that are independent of S1P and do not lead to release of the NH$_2$-terminal fragment.) When the SRD-12B cells expressed the KDEL-terminated S1P, SREBP-2 was cleaved to the nuclear form (lane 3), and cleavage was not inhibited by sterols (lane 4). Expression of the KDAS-terminated S1P also restored SREBP-2 processing (lane 5), but in this case the SREBP-2 cleavage was abolished by sterols (lane 6).

To follow the fate of the expressed S1P in the permanent cell lines, we subjected aliquots of whole-cell extracts and culture medium to electrophoresis, followed by immunoblotting with an antibody against the Myc epitope tag (Figure 6C). As expected, we visualized no Myc-tagged S1P in the parental SRD-12B cells (lanes 1 and 2). In the cells expressing the KDEL construct, we visualized all three forms (S1P-A, S1P-B, and S1P-C). In the cells expressing S1P-KDAS, we observed a strikingly different result. The cell extracts contained equivalent amounts of S1P-A and S1P-B, but there was much less S1P-C than we observed in the cells expressing S1P-KDEL. Instead, we found S1P-C in the culture medium of the S1P-KDAS cells (lanes 5 and 6). Sterols had no effect on the amount of S1P in either cell line. The data of Figure 6 are consistent with the hypothesis that S1P-KDEL is retained in the cells as a result of binding to the KDEL receptor and retrieval to the ER from the Golgi and that this retrieval leads to cleavage of SREBP-2 that cannot be blocked by sterols.

The experiments of Figure 7 were designed to determine whether S1P-KDEL obviates the requirement for SCAP and to confirm that cleavage by S1P-KDEL requires the RSVL recognition sequence in the luminal loop of SREBP-2. For this purpose, we transfected SCAP-deficient SRD-13A cells with a plasmid encoding wild-type HSV-tagged SREBP-2 or a mutant version containing an Ala in place of the Arg of the S1P recognition sequence, RSVL (Figure 7A). In the absence of any cotransfected plasmid, the SRD-13A cells failed to generate the nuclear form of SREBP-2 (middle panel, lanes 2 and 3). Cotransfection of a plasmid encoding SCAP restored SREBP-2 cleavage, and this was suppressed by sterols (lanes 4 and 5). Cotransfection of S1P-KDEL also restored cleavage, and this was no longer suppressed by sterols (lanes 6 and 7). The S1P-KDAS construct was unable to restore cleavage in SRD-13A cells (lanes 8 and 9). The S1P-KDEL plasmid did not stimulate cleavage of the R519A version of SREBP-2 (lanes 11 and 12). The bottom panel of Figure 7A shows that
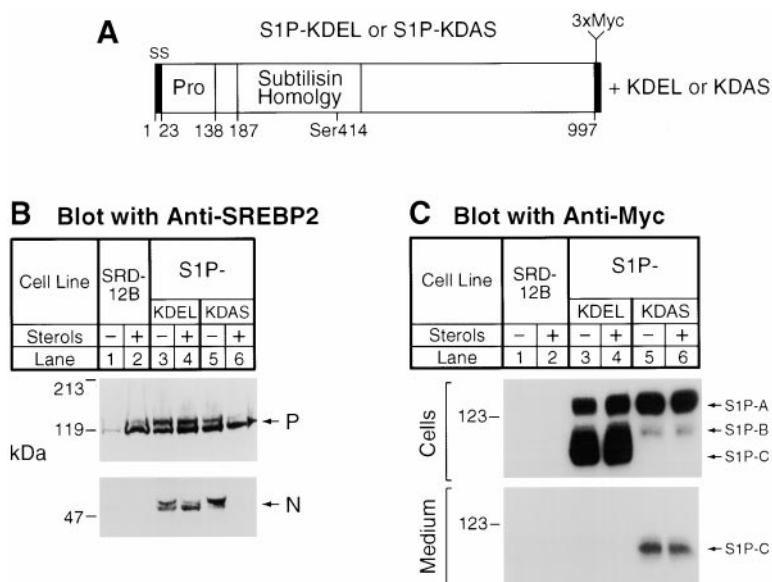
Figure 6. Stable Expression of S1P(1-997) Myc-KDEL in S1P-Deficient SRD-12B Cells Produces Unregulated Cleavage of SREBP-2

(A) Structure of S1P(1-997)Myc-KDEL and S1P(1-997)Myc-KDAS. The propeptide (Pro) and the subtilisin-like catalytic domain are boxed. The signal sequence (ss), catalytic site serine (Ser-414), Myc epitope tags, and the COOH-terminal four amino acids (KDEL or KDAS) are indicated. Amino acid numbers are shown below the diagram.

(B) Cleavage of SREBP-2 in SRD-12B cells stably transfected with pCMV-S1P(1-997) Myc-KDEL. Clones of SRD-12B cells that stably express either S1P(1-997)Myc-KDEL or S1P(1-997)Myc-KDAS were generated as described in the Experimental Procedures. SRD-12B cells (lanes 1 and 2) and SRD-12B cells stably transfected with pCMV-S1P (1-997)Myc-KDEL (lanes 3 and 4) or pCMV-S1P(1-997)Myc-KDAS (lanes 5 and 6) were cultured for 16 hr in medium A supplemented with 5% newborn calf lipoprotein-deficient serum and compactin/mevalonate in the absence or presence of sterols, and whole-cell extracts were prepared as described in the Experimental Procedures. Aliquots of cell extract (65 μg protein) were subjected to SDS-PAGE and immunoblotted with 5 μg/ml of monoclonal IgG-7D4 anti-SREBP-2. Filters were exposed to film for 5 s (top panel) and 30 s (bottom panel). P and N denote the precursor and cleaved nuclear forms of SREBP-2, respectively.

(C) Secretion of S1P(1-997)Myc-KDEL or S1P(1-997)Myc-KDAS in stably transfected SRD-12B cells. SRD-12B cells (lanes 1 and 2) and SRD-12B cells stably transfected with pCMV-S1P(1-997)Myc-KDEL (lanes 3 and 4) or pCMV-S1P(1-997)Myc-KDAS (lanes 5 and 6) were cultured for 16 hr in medium A supplemented with 5% newborn calf lipoprotein-deficient serum and compactin/mevalonate in the absence or presence of sterols. Samples of medium and whole-cell extracts were prepared as described in the Experimental Procedures. Aliquots of cell extracts (40 μg protein) and medium (corresponding to an equivalent amount of cells) were subjected to SDS-PAGE and immunoblotted with 2.5 μg/ml anti-Myc 9E10 antibody. Filters were exposed to film for 1 s. Molecular mass standards are expressed in kilodaltons.

S1P-C was detectable in membrane vesicles derived from the cells expressing S1P-KDEL, but not S1P-KDAS.

Figure 7B shows a similar transient transfection experiment in S1P-deficient SRD-12B cells. In the absence of a cotransfected plasmid, these cells failed to generate the nuclear form of HSV-tagged SREBP-2 (middle panel, lanes 2 and 3). Expression of wild-type S1P restored sterol-regulated cleavage (lanes 4 and 5). Expression of S1P-KDEL also restored cleavage, but there was no suppression by sterols (lanes 6 and 7). S1P-KDAS restored cleavage to a lesser extent, and sterol suppression was preserved (lanes 8 and 9). Neither S1P-KDEL nor S1P-KDAS permitted cleavage of the R519A version of SREBP-2 (lanes 11–14). Again, immunoblotting with anti-Myc revealed abundant S1P-C in the cells expressing S1P-KDEL, but much lower amounts in the cells expressing S1P-KDAS (lower panel of Figure 7B). These data indicate that expression of S1P-KDEL in the ER bypasses the SCAP requirement for SREBP cleavage.

Discussion

The current results support the hypothesis that cleavage of SREBPs at Site-1 requires vesicular transport of SREBPs from the ER to the Golgi and that this transport is blocked by sterols. The key to regulated transport is SCAP, which forms a tight complex with SREBPs. In sterol-depleted cells, SCAP escorts SREBP to a post-ER compartment that contains active S1P (see model in Figure 8A). In the presence of sterols, the SCAP/SREBP complex remains in the ER, and SREBP never reaches the compartment that contains active S1P.

Three lines of evidence support these conclusions. First, previous studies of SCAP glycosylation have shown that SCAP moves from the ER to the Golgi when cells are grown in the absence, but not the presence of sterols (Nohturfft et al., 1998b, 1999). Second, S1P-C, the predominant form of active S1P, is found in or near the Golgi apparatus, as shown by immunocytochemistry (Figures 3 and 4) and studies of glycosylation patterns (Figure 5). Third, retrograde transport of active S1P to the ER by two independent methods elicits unregulated SREBP processing and obviates the requirement for SCAP in SRD-13A cells (see Figure 8B).

The current findings with brefeldin A provide an explanation for the earlier findings of Ridgway and Lagace (Ridgway and Lagace, 1995), who observed that brefeldin A blocks sterol-mediated suppression of transcription in CHO cells. By redistributing active S1P from the Golgi to the ER, brefeldin A eliminates the need to transport SREBPs to the Golgi, allowing cleavage of SREBP in the presence as well as absence of sterols. This conclusion is supported by the demonstration that a similar effect can be achieved by attaching a KDEL signal to S1P, thereby trapping it in the ER.

The current studies also provide information about the Site-2 protease (S2P), which cleaves the NH_2-terminal intermediate form of SREBPs that is produced by cleavage at Site-1. If Site-1 cleavage normally occurs in a Golgi compartment, then S2P must be located in this compartment or a more distal compartment in the secretory pathway. When Site-1 cleavage takes place in the ER, as in brefeldin A–treated wild-type CHO cells, it is followed by Site-2 cleavage, thereby generating the
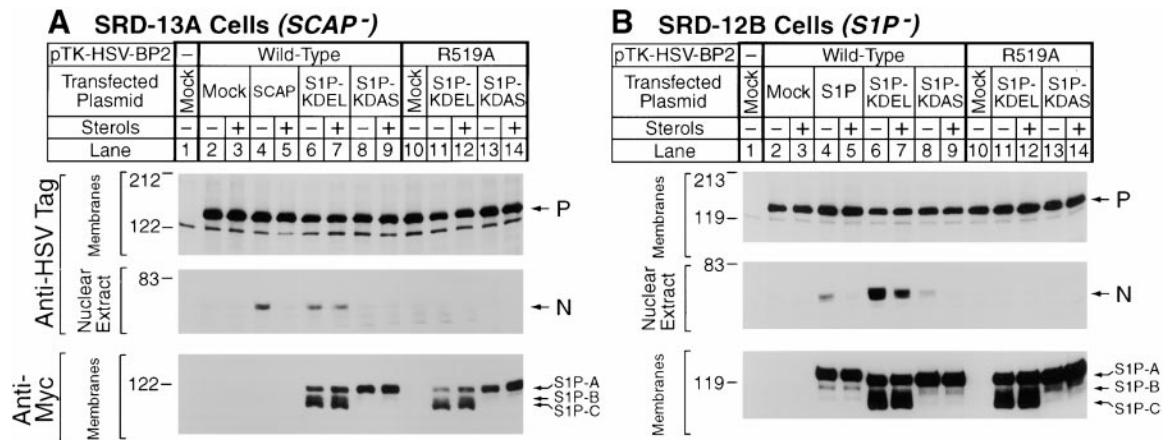
Figure 7. Transfected S1P(1-997)Myc-KDEL Rescues Cleavage of SREBP-2 in Cells Deficient in SCAP (A) and S1P (B)

(A) Immunoblot analysis of SREBP-2 cleavage in transfected SCAP-deficient SRD-13A cells. SRD-13A cells were set up on day 0 in medium B at 4×10^5 cells/60 mm dish and transfected on day 1 as described in the Experimental Procedures. The indicated plasmid encoding either wild-type (lanes 2–9) or mutant R519A (lanes 11–14) HSV-tagged SREBP-2 (1.75 μg/dish) was cotransfected into SRD-13A cells with empty vector (lanes 1–3, and 10), pTK-SCAP (0.05 μg/dish; lanes 4 and 5), pCMV-S1P(1-997)Myc-KDEL (0.25 μg/dish; lanes 6, 7, 11, and 12), or pCMV-S1P(1-997)Myc-KDAS (0.25 μg/dish; lanes 8, 9, 13, and 14) as indicated. The total amount of transfected DNA was adjusted to 3.75 μg/dish by the addition of pTK empty vector and pcDNA3 empty vector. Transfected cells were incubated for 18 hr in medium A supplemented with 5% newborn calf lipoprotein-deficient serum and compactin/mevalonate in the absence or presence of sterols, and the cells were harvested and fractionated as described in the Experimental Procedures. Aliquots of membranes (3 μg protein) and nuclear extract (15 μg protein) were subjected to SDS-PAGE and immunoblotted with 0.5 μg/ml IgG-HSV-Tag antibody (top and middle panels) or 2.5 μg/ml anti-Myc 9E10 antibody (bottom panel). Filters were exposed to film for 1 s. P and N denote the precursor and cleaved nuclear forms of SREBP-2, respectively. Molecular mass standards are expressed in kilodaltons.

(B) Immunoblot analysis of SREBP-2 cleavage in transfected S1P-deficient SRD-12B cells. SRD-12B cells were set up on day 0 in medium B at 4×10^5 cells/60 mm dish and transfected on day 1 as described in the Experimental Procedures. The indicated plasmid encoding either wild-type (lanes 2–9) or mutant R519A (lanes 11–14) HSV-tagged SREBP-2 (1.75 μg/dish) was cotransfected into SRD-12B cells with empty vector (lanes 1–3, and 10), pCMV-S1P-Myc (0.25 μg/dish; lanes 4 and 5), pCMV-S1P(1-997)Myc-KDEL (0.25 μg/dish; lanes 6, 7, 11, and 12), or pCMV-S1P(1-997)Myc-KDAS (0.25 μg/dish; lanes 8, 9, 13, and 14) as indicated. The total amount of transfected DNA was adjusted to 3.75 μg/dish by the addition of pTK empty vector and pcDNA3 empty vector. Cells were cultured and harvested as described in (A). Aliquots of membranes (4 μg protein) and nuclear extracts (11 μg protein) were subjected to SDS-PAGE and immunoblotted with 0.5 μg/ml IgG-HSV-Tag antibody (top and middle panels) or 2.5 μg/ml anti-Myc 9E10 antibody (bottom panel). Filters were exposed to film for 1 s. P and N denote the precursor and cleaved nuclear forms of SREBP-2, respectively. Molecular mass standards are expressed in kilodaltons.

nuclear form of SREBP (Figure 1). This implies either that some active S2P normally resides in the ER or that S2P is translocated there along with S1P following brefeldin A treatment. We also generated the nuclear form of SREBP after cleavage by the KDEL-terminated S1P (Figures 6 and 7). In this case, Site-2 cleavage might have been catalyzed by the fraction of active S2P that resides in the ER, or the cleaved intermediate form of SREBP might have left the ER and reached the Golgi compartment where S2P resides.
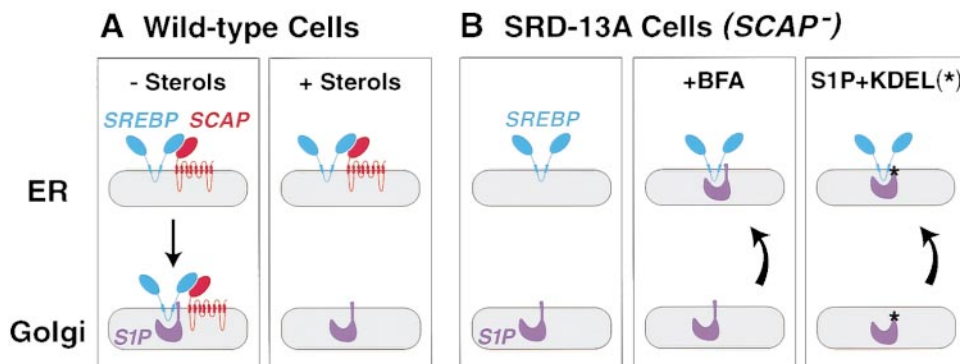

Figure 8. A Model Illustrating the Requirement of SCAP for Cleavage of SREBPs

(A) Wild-type cells. In the absence of sterols, SCAP transports SREBPs to the Golgi where S1P cleaves SREBPs. Transport of SCAP is blocked in the presence of sterols.

(B) SRD-13A cells (SCAP-deficient cells). In the absence of SCAP, SREBPs cannot gain access to active S1P, thus preventing cleavage (left panel). Treatment of cells with brefeldin A translocates S1P from the Golgi to the ER, restoring cleavage of SREBPs (middle panel). Retrieval of active, soluble S1P from the Golgi to the ER by the KDEL receptor also restores cleavage of SREBPs (right panel).

If active S1P and S2P are both located in the same compartment, this compartment is in or near the Golgi, as indicated by immunofluorescence studies (Figure 3). This compartment has the properties of the *cis-* or medial-Golgi stacks in that it collapses into the ER following treatment with brefeldin A (Figure 4). For this reason, we refer to this compartment as the Golgi. However, this compartment may not be composed of classic Golgi stacks. It might be part of the transitional network of tubules and vesicles that exists between the ER and Golgi. Further definition of the protease-containing compartment awaits the development of antibodies capable of visualizing S2P in nontransfected cells that express physiologic amounts of this protein. Cell fractionation studies and ultrastructural analysis by electron microscopy are also required.

Certain parallels exist between the role of SCAP in SREBP processing and the role played by presenilin in the processing of the amyloid precursor protein (APP), which is the only other animal cell protein that is clearly shown to be cleaved within a transmembrane region (Selkoe, 1996). Presenilin, like SCAP, is a polytopic membrane protein that is required for proteolytic processing of APP (Haass and De Strooper, 1999). Some (Weidemann et al., 1997; Xia et al., 1997), but not all (Thinakaran et al., 1998) studies show that presenilin forms a complex with full-length APP prior to proteolytic cleavage. In addition, presenilin is necessary in order for APP to be transported to the cellular compartment where the γ-secretase resides (Naruse et al., 1998). These findings have direct parallels with SCAP. A corollary of this hypothesis is that the absence of presenilin prevents APP from reaching the compartment where the γ-secretase resides (Naruse et al., 1998). This is analogous to the fate of SREBP in SCAP-deficient cells.

Although SCAP and presenilin share certain functions, the proteins have no sequence resemblance. Moreover, they facilitate different reactions. SCAP facilitates the first cleavage of SREBP, which occurs in the luminal domain of SREBP. Presenilin facilitates the second cleavage of APP, namely the intramembrane cleavage that follows the luminal cleavage. Indirect evidence suggests that presenilin may be an aspartyl protease that cleaves APP at the γ site (Wolfe et al., 1999). Like Site-2 in SREBP, the γ cleavage site of APP is located within a transmembrane domain. However, there is no evidence that SCAP is a protease, and indeed we have isolated a protein with the characteristics of a hydrophobic zinc metalloprotease that appears to carry out Site-2 cleavage of SREBPs (Rawson et al., 1997).

In a more general sense, the results with SCAP and presenilin point out the important role of regulated vesicular transport in carrying membrane proteins to their sites of processing. The postulated transport roles of SCAP and presenilin raise the possibility that polytopic membrane proteins may function as escort molecules that carry specific proteins to their sites of processing. Schekman and colleagues have postulated the existence of these molecular sorters in yeast (Springer et al., 1999). It will be important to determine whether escort proteins like SCAP or presenilin target proteins to dedicated vesicles, or whether they simply select individual proteins for incorporation into a single set of common

carrier vesicles that are targeted to a particular organelle. This situation would be analogous to the function of cell surface clathrin-coated pits, which are common carriers whose cargo is selected by sorting molecules.

Experimental Procedures

Materials
We obtained monoclonal anti-HSV-Tag (IgG1) from Novagen, monoclonal anti-Myc (clone 9E10) from Roche Molecular Biochemicals, horseradish peroxidase-conjugated donkey anti-mouse and anti-rabbit IgG (affinity-purified) from Jackson Immunoresearch Laboratories, horseradish peroxidase-conjugated donkey anti-rabbit whole antibody from Amersham Pharmacia Biotech, glycosidases from New England Biolabs, and brefeldin A from Calbiochem. The following recombinant expression plasmids were previously described: pTK-HSV-BP2 (WT and R519A), encoding wild-type and mutant HSV-tagged human SREBP-2 (Hua et al., 1996b); pTK-SCAP, encoding hamster SCAP (Nohturfft et al., 1998b); and pCMV-Myc-S1P, pCMV-S1P-Myc, and pCMV-S1P, encoding hamster S1P (Espenshade et al., 1999). Other reagents were obtained from sources as described previously (Wang et al., 1994). Newborn and fetal calf lipoprotein-deficient sera (d > 1.215 mg/ml) were prepared as described (Goldstein et al., 1983).

Cell Culture
Cells were maintained in monolayer culture at 37°C in 8%-9% CO_2. CHO-7 cells are a clone of CHO-K1 cells selected for growth in lipoprotein-deficient serum (Metherall et al., 1989). CHO/pS2P cells (Rawson et al., 1998) are a clone of CHO-7 cells stably transfected with pCMV-HSV-S2P, a plasmid that encodes human Site-2 protease under control of the cytomegalovirus (CMV) promoter enhancer. SRD-12B cells (deficient in Site-1 protease) and SRD-13A cells (deficient in SCAP) are previously described cholesterol and unsaturated fatty acid auxotrophs derived from γ-irradiated CHO/pS2P cells (Rawson et al., 1998, 1999). In experiments using SRD-12B or SRD-13A cells, the parental line, CHO/pS2P, was used as a control. Stock cultures of CHO-7 cells were maintained in medium A (1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle medium containing 100 U/ml penicillin and 100 μg/ml streptomycin sulfate) supplemented with 5% (v/v) newborn or fetal calf lipoprotein-deficient serum. Stock cultures of CHO/pS2P cells were maintained in medium A supplemented with 5% fetal calf lipoprotein-deficient serum, 2 μM compactin, and 500 μg/ml G418. Stock cultures of SRD-12B and SRD-13A cells were maintained in medium B (medium A supplemented with 5% (v/v) fetal calf serum, 5 μg/ml cholesterol, 1 mM sodium mevalonate, and 20 μM sodium oleate).

Construction of pCMV-S1P(1-997)Myc-KDEL and pCMV-S1P(1-997)Myc-KDAS
The expression vector pCMV-S1P(1-997)Myc-KD*XX* encodes amino acids 1-997 of hamster S1P followed by three tandem copies of the c-Myc epitope tag (GGRS<u>EQKLISEEDL</u>NG<u>EQKLISEEDL</u>NG<u>EQKLISEEDL</u>NSSGR) and the amino acids KDEL or KDAS under the control of the CMV promoter enhancer. To generate these plasmids, we used the QuikChange site-directed mutagenesis kit (Stratagene) to mutagenize pCMV-S1P (Sakai et al., 1998b) using the following oligonucleotides: 5′-GCCGCTACAACCAAGAGGGCGGCCGCAAG GACGAGCTGTGATAAGTGGGCCAGACCATCCC-3′ for the KDEL plasmid and 5′-GCCGCTACAACCAAGAGGGCGGCCGCAAGGAC GCCTCCTGATAAGTGGGCCAGACCATCCC-3′ for the KDAS plasmid. Following mutagenesis, a 120 bp fragment flanked by NotI sites encoding three tandem copies of the c-Myc epitope (Sakai et al., 1998b) was inserted into the NotI site of the newly created plasmids. In all plasmid constructions, mutations were confirmed by sequencing the relevant regions.

Stable Transfection of SRD-12B Cells
On day 0, cholesterol auxotrophic SRD-12B cells were plated at a density of 4×10^5 cells/60 mm dish in medium B. On day 1, cells were transfected with 0.5 μg of either pCMV-S1P(1-997)Myc-KDEL or pCMV-S1P(1-997)Myc-KDAS plus 2.5 μg of pcDNA3 (Invitrogen)

per dish using the lipofectamine method as described previously (Sakai et al., 1998b). On day 2, the medium was switched to medium A supplemented with 5% fetal calf lipoprotein-deficient serum (containing no added cholesterol). The medium was changed as needed for 12 days until individual colonies were visible. Stable expression of either S1P(1-997)Myc-KDEL or S1P(1-997)Myc-KDAS permitted growth of SRD-12B cells in the absence of exogenous cholesterol. Single-cell clones that stably expressed S1P(1-997)Myc-KDEL and S1P(1-997)Myc-KDAS were isolated by limiting dilution and screened for S1P expression by immunoblotting with anti-Myc (clone 9E10) monoclonal antibody. Two cell lines expressing equivalent levels of S1P(1-997)Myc-KDEL and S1P(1-997)Myc-KDAS were selected for further studies and designated S1P-KDEL and S1P-KDAS cells, respectively.

For experiments using SRD-12B stable cell lines, cells were set up on day 0 at a density of 7×10^5 cells/10 cm dish in medium B. On day 1, the medium was switched to medium A supplemented with 5% newborn calf lipoprotein-deficient serum, 50 μM compactin, and 50 μM sodium mevalonate in the absence or presence of sterols (1 μg/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol added in a final concentration of 0.2% ethanol). After 15 hr, N-acetyl-leucinal-leucinal norleucinal (ALLN) was added to a final concentration of 25 μg/ml for 1 hr prior to harvest. To prepare whole-cell extracts, cell monolayers were washed three times in cold Dulbecco's phosphate-buffered saline containing 1 mM sodium EDTA and 1 mM sodium EGTA. To lyse the cells, 0.25 ml of SDS lysis buffer containing 1$\times$ protease inhibitor cocktail (1 mM dithiothreitol, 1 mM PMSF, 0.5 mM Pefabloc, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 25 μg/ml ALLN, and 10 μg/ml aprotinin) was added to each dish. Cell lysate was passed five times through a 22.5-gauge needle and five times though a 25-gauge needle. Whole-cell lysates were mixed with 5$\times$ SDS loading buffer and boiled for 5 min prior to SDS-PAGE. Aliquots of the culture medium were collected, precipitated with acetone, and resuspended in SDS lysis buffer as previously described (Espenshade et al., 1999).

Immunoblot Analysis of SREBP Processing
On day 0, SRD-12B and SRD-13A cells were set up at the indicated density in medium B in 60 mm dishes. On day 1, cells were transfected with 4 μg of DNA/dish using 12 μl of Fugene 6 reagent (Roche Molecular Biochemicals) in a final volume of 0.2 ml. Transfection was performed as previously described (Rawson et al., 1999). Cells were incubated at 37°C for 16–24 hr in medium A supplemented with 5% fetal calf serum. On day 2, the medium was removed, and the cells were cultured in medium A supplemented with 5% newborn calf lipoprotein-deficient serum, 50 μM compactin, and 50 μM sodium mevalonate in the absence or presence of sterols (1 μg/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol added in a final concentration of 0.2% ethanol). After incubation at 37°C for 16 hr, the cells received ALLN at a final concentration of 25 μg/ml. Following a 1 hr incubation, the cells were harvested and processed as previously described (Sakai et al., 1996) with minor modifications. Harvested cells were resuspended in 0.4 ml of buffer A (10 mM HEPES-KOH at pH 7.4, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 250 mM sucrose), passed through a 22.5-gauge needle 30 times, and centrifuged at 1000 g for 7 min at 4°C. The 1000 g pellet was resuspended in 0.1 ml of buffer B (20 mM HEPES-KOH at pH 7.6, 2.5% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl$_2$, 1 mM sodium EDTA, 1 mM sodium EGTA), rotated at 4°C for 1 hr, and centrifuged at 10^5 g for 30 min at 4°C in a Beckman TLA 100.2 rotor. Fractionation buffers contained 1$\times$ protease inhibitor cocktail. The supernatant from this spin was designated the nuclear extract. The supernatant from the original 1000 g spin was used to prepare the membrane fraction by centrifugation at 2×10^4 g for 15 min at 4°C. The resulting membrane pellets were resuspended in 0.1 ml SDS-lysis buffer (Espenshade et al., 1999).

Protein concentration in nuclear extract and membrane fractions was measured using the BCA Kit (Pierce), and the samples were mixed with 5$\times$ SDS loading buffer (Bollag and Edelstein, 1991). After boiling for 5 min, the proteins were subjected to SDS-PAGE and transferred to Hybond C-Extra nitrocellulose filters (Amersham). The filters were incubated with the antibodies described in the figure legends. Bound antibodies were visualized with peroxidase-conjugated affinity-purified donkey anti-mouse or anti-rabbit IgG using the SuperSignal CL-HRP substrate system (Pierce) according to the manufacturer's instructions. Gels were calibrated with prestained molecular mass markers (Bio-Rad). Filters were exposed to X-Omat Blue XB-1 film (Kodak) at room temperature for the indicated times.

Glycosidase Sensitivity of S1P
SRD-12B and CHO/pS2P cells were cultured in medium A supplemented with 5% newborn calf lipoprotein-deficient serum, 50 μM compactin, and 50 μM mevalonate in the absence or presence of sterols (1 μg/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol added in a final concentration of 0.2% ethanol). After 16 hr, ALLN was added to a final concentration of 25 μg/ml, and the cells were harvested 1 hr later. Membrane and nuclear extract fractions were prepared as described above. Aliquots of solubilized membrane proteins (15 μg) were treated with either peptide N-glycosidase F or endoglycosidase H (endo H) as described previously (Sakai et al., 1998b).

Indirect Immunofluorescence Microscopy
CHO/pS2P and SRD-12B cells were set up on glass coverslips in medium B. Cells were washed in buffer C containing 0.1 M sodium phosphate at pH 7.4, 0.15 M NaCl, 4 mM KCl, 2 mM MgCl$_2$, and 0.02% (w/v) sodium azide and then fixed and permeabilized by incubating for 10 min in solution containing 60% (v/v) methanol, 10% (v/v) glacial acetic acid, and 30% (v/v) 1,1,1-trichloroethane. After a brief rinse with buffer C, cells were incubated for 30 min at room temperature in blocking buffer (0.1 M Tris–HCl at pH 9.0, 1% [w/v] bovine serum albumin, 0.1 M NaCl, 0.02% [w/v] NaN$_3$). Cells were incubated at 4°C overnight with affinity-purified anti-S1P rabbit polyclonal antibody (20 μg/ml in blocking buffer). Primary antibodies were localized by incubating cells for 2 hr in 20 μg/ml affinity-purified, goat anti-rabbit IgG conjugated to Alexa 488 (Molecular Probes, Inc.) in blocking buffer. For Golgi compartment staining, rhodamine-labeled wheat germ agglutinin (Vector Laboratories, Inc.) was added during the second antibody incubation at a concentration of 5 μg/ml. Cells were washed three times after each antibody or lectin incubation and analyzed with a Leica TCS SP confocal microscope. S1P-specific antibodies were isolated from rabbit serum by affinity chromatography using a cross-linked agarose resin conjugated to the two immunizing synthetic peptides (Espenshade et al., 1999; Harlow and Lane, 1999). Peptides were conjugated to SulfoLink Coupling Gel (Pierce) according to the manufacturer's instructions. For the brefeldin A experiment, cells were treated with brefeldin A at a final concentration of 2 μg/ml in 0.2% methanol for 1 hr prior to fixation of cells.

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