Reconstitution of Sterol-regulated Endoplasmic Reticulum-to-Golgi Transport of SREBP-2 in Insect Cells by Co-expression of Mammalian SCAP and Insigs*

In mammalian cells, membrane-bound sterol regulatory element-binding proteins (SREBPs) are transported from ER to Golgi where they are processed proteolytically to generate soluble transcription factors that activate lipid synthesis. ER-to-Golgi transport requires SCAP, a sterol-regulated escort protein. In sterol-treated cells, the SCAP/SREBP complex binds to Insig-1 or Insig-2, which retains the complex in the ER, blocking SREBP processing and decreasing lipid synthesis. In Drosophila cells, the endogenous SCAP/SREBP complex is transported to Golgi, but transport is blocked by phosphatidylethanolamine instead of sterols. Here, we show that mammalian SREBP-2 is not transported to Golgi when expressed in Drosophila cells. Transport requires co-expression of mammalian SCAP. Sterols block transport of the mammalian SCAP/SREBP-2 complex, but only when mammalian Insig-1 or -2 is co-expressed. These reconstitution studies define SCAP and Insig as the minimal requirements for sterol-regulated transport of SREBPs from ER to Golgi. They indicate that insect cells can respond to sterols when proper regulatory proteins are expressed.

In mammalian cells, sterols block the movement of sterol regulatory element-binding proteins (SREBPs)1 from the endoplasmic reticulum (ER) to the Golgi complex, thereby turning off the synthesis of membrane lipids, including cholesterol and unsaturated fatty acids (1, 2). SREBPs are transcription factors that are inserted co-translationally into ER membranes by virtue of two membrane-spanning helices. Soon after their synthesis, the SREBP's bind to SREBP cleavage-activating protein (SCAP), a polytopic membrane protein that escorts the SREBPs to the Golgi complex. In the Golgi, the SREBP is processed by Site-1 protease (S1P), which cleaves the SREBP in the luminal loop between the two transmembrane helices. This cleavage generates an NH2-terminal intermediate fragment that contains the transcription factor domain. The intermediate fragment remains bound to membranes through a single membrane-spanning helix. At this point, a second enzyme, Site-2 protease (S2P), cleaves the membrane-spanning helix, releasing the NH2-terminal domain so that it can enter the nucleus and activate genes involved in lipid synthesis and uptake. When cholesterol accumulates in ER membranes, the SCAP/SREBP complex fails to exit the ER, proteolytic processing is abrogated, and lipid synthesis declines. This feedback mechanism assures a constant membrane composition under conditions of varying cholesterol supply (1, 2).

Retention of the SCAP/SREBP complex in the ER is mediated by the sterol-dependent binding of the complex to one of two ER retention proteins designated Insig-1 and Insig-2 (3, 4). The Insigs bind to the membrane-associated domain of SCAP, which comprises eight transmembrane helices. Point mutations at any one of three positions within this domain block the binding of SCAP to Insigs (3, 4) and prevent sterols from inhibiting SREBP processing (5–7).

Recent studies of cultured Drosophila Schneider S2 cells have revealed four differences in the regulation of SREBP processing in insects as compared with mammals (8, 9). First, a regulatory role for Drosophila SREBP in controlling cholesterol synthesis is ruled out by the well-established observation that insects cannot synthesize cholesterol (10). Second, in contrast to mammals that produce three different SREBP isoforms that control synthesis of both cholesterol and fatty acids (1, 11), Drosophila produces only one SREBP that controls synthesis of fatty acids, but not cholesterol (8). Third, whereas Drosophila cells produce an orthologue of SCAP, they lack a recognizable Insig gene. And fourth, processing of SREBP in cultured Drosophila cells is inhibited by a lipid derived from palmitate and ethanolamine, most likely phosphatidylethanolamine, and not by sterols (9).

In the current studies, we reconstitute sterol-mediated regulation of SREBP processing by expressing mammalian proteins in cultured Drosophila S2 cells. For this purpose, we transfected the Drosophila cells with cDNAs encoding human SREBP-2, hamster SCAP, and human Insig-1 or human Insig-2. The human SREBP-2 binds to hamster SCAP and is transported to the Golgi as determined by the processing of SCAP carbohydrates to an endoglycosidase H (endo H)-resistant form. In the Golgi, the human SREBP-2 is processed by Drosophila S1P, producing a membrane-bound intermediate. Transport to the Golgi and proteolytic processing is blocked by sterols, but only when hamster Insig-1 or -2 is co-expressed. These results demonstrate that SREBPs, SCAP, and Insigs are sufficient to reconstitute sterol-regulated transport from ER to Golgi in an insect cell whose own SREBP is not regulated by sterols.

Received for publication, June 18, 2003, and in revised form, July 2, 2003
Published, JBC Papers in Press, July 3, 2003, DOI 10.1074/jbc.M306476200

Irina Y. Dobrosotskaya, Joseph L. Goldstein‡, Michael S. Brown‡, and Robert B. Rawson
From the Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9046

* This work was supported by research grants from the National Institutes of Health (HL20948), Perot Family Foundation, Moss Heart Foundation, and W. M. Keck Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence may be addressed: Dept. of Molecular Genetics, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Rm. L5.238, Dallas, TX 75390-9046. Tel.: 214-648-2141; Fax: 214-648-8804; E-mail: joe.goldstein@utsouthwestern.edu (to J. L. G.) or mike.brown@utsouthwestern.edu (to M. S. B.).

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 37, Issue of September 12, pp. 35837–35843, 2003
Printed in U.S.A.

This paper is available on line at http://www.jbc.org

This is an Open Access article under the CC BY license.
This paper is available on line at http://www.jbc.org

35837
Expression Plasmids—The following plasmids were constructed by subcloning the indicated open reading frame from mammalian expression vectors into pAc5.1/V5-HisB (Invitrogen), a Drosophila expression vector under the control of Drosophila actin 5C promoter: pAc-humInsig-1-Myc encodes the open reading frame of human Insig-1 tagged with six copies of the Myc epitope at its COOH terminus subcloned from pCMV-Insig-1-Myc; pAc-humInsig-2-Myc encodes the open reading frame of human Insig-2 tagged with six copies of the Myc epitope at its COOH terminus amplified by polymerase chain reaction (PCR) from pCMV-Insig-1-Myc; pAc-hamSCAP encodes the open reading frame of hamster SCAP subcloned from pCMV-SCAP (12); pAc-hamSCAP-T7(Y298C), pAc-hamSCAP-D443N, and pAc-hamSCAP-L315F encode the open reading frames of hamster SCAP containing the respective point mutations subcloned from pTK-HSV-SCAP-D443N and pTK-HSV-SCAP-L315F as indicated. The PCR product was digested using the PCR Optimizer™ kit (Invitrogen), and subcloned into pAc5.1/V5-HisB (Invitrogen), a Drosophila expression vector under control of the Drosophila actin 5C promoter.

pAc-Myc-dSCAP encodes the open reading frame of Drosophila SCAP tagged with three Myc tags at its NH2-terminal end, under control of the Drosophila actin 5C promoter. To generate pAc-Myc-dSCAP, a fragment containing the first 1093 nucleotides of the dSCAP coding region was generated by RT-PCR from Drosophila w1118 strain poly-A+ RNA (primers: 5′, TGTGGCGATTATCGTCAATGCGGCGCCGACAGATA-T; 3′, TCGGCTTTCTCATGTTTCTGGTCGCAGGCCGACAAGTGTC) using the PCR Optimizer™ kit (Invitrogen), and subcloned into pAc5.1/V5-HisB via EcoRI (5′) and XhoI (3′) restriction sites. The remainder of the open reading frame was amplified from the dSCAP cDNA (8) (primers: 5′, TTCATCTCTAGAGCGCTTTAATGGCACTTGTCTGCCA; 3′, GCTGAGCTGCACCCAAGGGGCTGG). The PCR product was digested with HindIII and XhoI endonucleases and subcloned into the above plasmid to yield the complete open reading frame of dSCAP. A NorI site was then introduced into the NH2-terminal end of the construct to allow insertion of three tandem Myc tags, which were excised from the NotI-containing pCMV-Myc-S1P (14).

pDS-HSV-dSREBP encodes the open reading frame of dSREBP, tagged at its NH2 terminus with an HSV tag under control of the Drosophila DS47 promoter as described (8).

**EXPERIMENTAL PROCEDURES**

Materials—Unless otherwise stated, all reagents and materials were prepared or obtained from sources as previously described (6, 8, 9).

Expression Plasmids—The following plasmids were constructed by subcloning the indicated open reading frame from mammalian expression vectors into pAc5.1/V5-HisB (Invitrogen), a Drosophila expression vector under the control of Drosophila actin 5C promoter: pAc-humInsig-1-Myc encodes the open reading frame of human Insig-1 tagged with six copies of the Myc epitope at its COOH terminus subcloned from pCMV-Insig-1-Myc; pAc-humInsig-2-Myc encodes the open reading frame of human Insig-2 tagged with six copies of the Myc epitope at its COOH terminus amplified by polymerase chain reaction (PCR) from pCMV-Insig-1-Myc; pAc-hamSCAP encodes the open reading frame of hamster SCAP subcloned from pCMV-SCAP (12); pAc-hamSCAP-Y298C, pAc-hamSCAP-D443N, and pAc-hamSCAP-L315F encode the open reading frames of hamster SCAP containing the respective point mutations subcloned from pTK-HSV-SCAP-D443N and pTK-HSV-SCAP-L315F as indicated. The PCR product was digested using the PCR Optimizer™ kit (Invitrogen), and subcloned into pAc5.1/V5-HisB (Invitrogen), a Drosophila expression vector under control of the Drosophila actin 5C promoter.

pAc-Myc-dSCAP encodes the open reading frame of Drosophila SCAP tagged with three Myc tags at its NH2-terminal end, under control of the Drosophila actin 5C promoter. To generate pAc-Myc-dSCAP, a fragment containing the first 1093 nucleotides of the dSCAP coding region was generated by RT-PCR from Drosophila w1118 strain poly-A+ RNA (primers: 5′, TGTGGCGATTATCGTCAATGCGGCGCCGACAGATA-T; 3′, TCGGCTTTCTCATGTTTCTGGTCGCAGGCCGACAAGTGTC) using the PCR Optimizer™ kit (Invitrogen), and subcloned into pAc5.1/V5-HisB via EcoRI (5′) and XhoI (3′) restriction sites. The remainder of the open reading frame was amplified from the dSCAP cDNA (8) (primers: 5′, TTCATCTCTAGAGCGCTTTAATGGCACTTGTCTGCCA; 3′, GCTGAGCTGCACCCAAGGGGCTGG). The PCR product was digested with HindIII and XhoI endonucleases and subcloned into the above plasmid to yield the complete open reading frame of dSCAP. A NorI site was then introduced into the NH2-terminal end of the construct to allow insertion of three tandem Myc tags, which were excised from the NotI-containing pCMV-Myc-S1P (14).

pDS-HSV-dSREBP encodes the open reading frame of dSREBP, tagged at its NH2 terminus with an HSV tag under control of the Drosophila DS47 promoter as described (8).

Drosophila S2 Cells—Stock cultures of Drosophila S2 cells were maintained in medium A (Schneider’s Drosophila medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate) at 23 °C as previously described (8). Cells were set up for experiments on day 0 in medium A at a density of 6 × 105 cells per 37-mm dish. Several hours later, the cells were transfected in Drosophila-SFM medium (Invitrogen) using 10 μl per dish of Cellfectin™ Reagent (Life Technologies) according to the manufacturer’s instructions. The amount of DNA was adjusted to 1.5 μg per dish with empty vector DNA. On day 1, cells were refed with 2 ml/dish of medium B (IPL-41 medium supplemented with 5% of heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate). On day 3, the cells were washed once with phosphate-buffered saline (PBS) and refed with 3 ml/dish of medium C (IPL-41 medium supplemented with 5% of heat-inactivated delipidated...
fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate) containing various additions of sterols (25-hydroxycholesterol plus cholesterol) and/or sodium palmitate plus ethanolamine. The mixture of sterols was added at a final concentration of 0.2% (v/v) ethanol; the mixture of sodium palmitate bound to fatty acid-free bovine serum albumin (15) and ethanolamine was added at a final concentration of 0.2% (w/v) bovine serum albumin. All control cultures received additions of 0.2% ethanol and/or 0.2% bovine serum albumin.

Proteolytic Processing of SREBPs—On day 0, cells were plated and transfected as described above. On day 3, cells were treated as described above and in figure legends, after which they were harvested by scraping in phosphate-buffered saline. For preparation of whole-cell lysates, the cell pellets from two replicate dishes were combined and solubilized in buffer D (100 mM sodium chloride, 1.5 mM magnesium chloride; 50 mM Hepes-KOH at pH 7.4, and 0.5% (w/v) CHAPS) containing protease inhibitors as described (8). The insoluble residue was removed by centrifugation for 5 min at 20,000 × g at 4 °C. For preparation of subcellular fractions, the cell pellets from six replicate dishes were combined and treated as described (8).

Immunoprecipitation—The cell pellets from six replicate dishes were combined and resuspended in 1 ml of buffer D supplemented with protease inhibitors as described above. Cells were disrupted by passing 20 times through a 22-gauge needle, and the suspension was incubated with constant rotation at 4 °C for 1 h. After the lysates were cleared by centrifugation at 20,000 × g for 20 min at 4 °C, the resulting supernatants were subjected to immunoprecipitation as described (7) with the following minor modifications: the concentration of control rabbit polyclonal IgG or rabbit polyclonal anti-Myc IgG was 25 μg/ml and the amount of Protein A/G PLUS Agarose was 60 μl/tube.

Endo H Assay—The number of N-linked carbohydrate chains on SCAP was analyzed by the endoglycosidase H (endo H) assay as described (6). Briefly, aliquots of membranes (80 μg of protein) were incubated for 30 min at 30 °C with 17 μg/ml trypsin (Sigma), and proteolysis was stopped with soybean trypsin inhibitor, followed by addition of 0.5% SDS and 1% 2-mercaptoethanol and boiling for 10 min. One-half of each sample was then incubated in the absence or presence of 10.4 units/ml endo H (New England Biolabs) for 12 h at 37 °C, after which the samples were subjected to SDS-PAGE (8–12% gradient gel) and immunoblot analysis with IgG-9D5 (anti-SCAP), and exposed to film for 30 s.

SDS-PAGE and Immunoblot Analysis—Subcellular fractions and cell lysates were subjected to 8% SDS-PAGE and analyzed by immunoblotting as described (8). The following antibodies were used for immunoblot analysis: 50 ng/ml anti-HSV IgG (Novagen) to detect epitope-tagged human and Drosophila SREBP; 0.1 μg/ml anti-Myc IgG-9E10 to detect epitope-tagged human Insig-1 and Insig-2 (3); 2 μg/ml anti-Myc IgG-9E10 to detect epitope-tagged Drosophila SCAP; and 5 μg/ml IgG-9D5 to detect hamster SCAP (12). Filters were exposed to film for 1–30 s.

RESULTS

Fig. 1A shows a series of immunoblots designed to follow the processing of transfected HSV-tagged Drosophila SREBP in Drosophila S2 cells. As shown previously (8), Drosophila SREBP is carried to the Golgi by endogenous SCAP and processed by endogenous SIP and S2P to a cleaved form that is present in nuclear extracts (lane 2). Processing was blocked by incubation with palmitate plus ethanolamine (lane 4), but not with a mixture of cholesterol and 25-hydroxycholesterol (sterols, lane 5). There was no change in processing of Drosophila SREBP when mammalian Insig-1 was co-expressed (lanes 6–8).

Strikingly different results were obtained when we examined the processing of mammalian SREBP in the Drosophila S2 cells (Fig. 1B). When human SREBP-2 was expressed alone, there was no processing of the precursor (lane 2). When hamster SCAP was co-expressed, we observed the processing of SREBP-2 to a membrane-bound cleaved fragment whose size corresponded to the product of cleavage at Site-1. We refer to this band as the intermediate fragment (lane 3). Cleavage to the intermediate fragment was not suppressed by palmitate plus ethanolamine, nor by sterols (lanes 4 and 5). Co-expression of human Insig-1 did not affect processing to the intermediate fragment in the absence of added lipids (lane 6), but it did sensitize this processing to partial inhibition by palmitate plus ethanolamine (lane 7) and near-complete inhibition by sterols (lane 8). Unlike the intermediate form of Drosophila SREBP, the intermediate form of human SREBP-2 was apparently not recognized by the endogenous Drosophila S2P, and thus no nuclear form of human SREBP-2 was generated.

To be certain that the Drosophila SIP was indeed responsible for the generation of the intermediate form of human SREBP-2 in Fig. 1B, we transfected a mutant human SREBP-2 that contains a substitution of alanine for arginine at residue 519, a substitution in a recognition sequence that is required for processing by mammalian and Drosophila SIP (8, 16). As shown in Fig. 2, very little of the intermediate form was generated from the mutant R519A version of SREBP-2 as compared with wild-type SREBP-2 (lanes 5 versus 3).

Fig. 3 compares the ability of human Insig-1 and Insig-2 to confer sterol-mediated regulation upon the processing of hu-
man SREBP-2 in Drosophila cells. In cultured mammalian cells, cholesterol is a weak regulator of SREBP processing when added to the culture medium in ethanol. Full regulation requires supplementation with a hydroxylated derivative of cholesterol, such as 25-hydroxycholesterol, which enters cells more readily than cholesterol (3, 17). In the experiment of Fig. 3A, all of the medium contained 10 μg/ml of cholesterol. In the presence of SCAP but the absence of Insig, 25-hydroxycholesterol failed to inhibit the cleavage of human SREBP-2 as revealed by the persistence of the membrane-bound intermediate fragment (Fig. 3A, lanes 2–6). When Insig-1 was expressed, there was a moderate reduction in SREBP-2 cleavage in the absence of 25-hydroxycholesterol (compare lanes 7 and 2). Addition of 25-hydroxycholesterol led to a nearly complete inhibition of cleavage (lanes 8–11). Expression of Insig-2 did not reduce the amount of the SREBP-2 intermediate fragment in the absence of 25-hydroxycholesterol (lane 12), but it sensitized the cleavage to inhibition by the sterol (lanes 13–16).

To determine whether the 25-hydroxycholesterol-independent inhibition of SREBP-2 cleavage by Insig-1 (Fig. 3A, lane 7) was caused by the presence of cholesterol in the culture medium, we performed the experiment of Fig. 3B. The results show that cholesterol alone does not inhibit cleavage of human SREBP-2 in either the absence or presence of Insig-1 or Insig-2 (lanes 3, 7, and 11).

Several point mutations that render hamster SCAP resistant to sterols have been identified (5–7). We next tested whether these mutant forms of mammalian SCAP would behave in a similar fashion in Drosophila cells. Hamster SCAP with a tyrosine to cysteine substitution at position 298 (Y298C) fails to interact with Insigs, and therefore it carries SREBPs to the Golgi for cleavage even in the presence of sterols (3). Fig. 4A shows that this functional defect persists when the mutant mammalian SCAP is expressed in Drosophila cells. Thus, 25-hydroxycholesterol suppressed human SREBP-2 cleavage in cells that expressed Insig-1 and wild-type SCAP (lanes 6–10), but not in cells that expressed SCAP(Y298C) (lanes 16–20). We noted that the level of Insig-1 was lower in cells expressing SCAP(Y298C) than in cells expressing wild-type SCAP (compare the Insig blots in lanes 16–20 and 6–10). To make certain that the 25-hydroxycholesterol resistance of the mutant hamster SCAP was not attributable to the lower level of Insig-1, we transfected the cells with a higher amount of Insig-1 plasmid that produced an Insig level that was equal to that in the cells expressing wild-type SCAP. The resistance to Insig-1 was still apparent (lanes 21–25).
Sterol-regulated co-immunoprecipitation of mammalian Insig, SCAP, and SREBP in Drosophila S2 cells. On day 0, S2 cells were set up and transfected with the indicated combinations of pAc-humInsig-1-Myc (0.3 μg), pAc-humInsig-2-Myc (0.3 μg), pAc-ham-SCAP (0.05 μg), and pDS-HSV-humSREBP-2 (0.3 μg). On day 3, cells were refed with medium C containing either 10 μg/ml of cholesterol plus 1 μg/ml of 25-hydroxycholesterol (S) or 100 μm sodium palmitate plus 100 μm ethanolamine (PE) as indicated. Eight hours later, cells were harvested and subjected sequentially to immunoprecipitation, SDS-PAGE, and immunoblotting as described under “Experimental Procedures.” The amounts of analyzed sample relative to the total sample was 0.5 for pellet and 0.05 for supernatant (Sup.).

Two other point mutations in mammalian SCAP (L315F and D443N) also interfere with Insig interactions. Fig. 4B shows that cleavage of SREBP-2 was also resistant to 25-hydroxycholesterol in Drosophila cells that expressed either of these two mutant forms of SCAP. This figure also shows that all three mutant forms of SCAP conveyed resistance to cleavage inhibition by palmitate plus ethanolamine, although this resistance was not as complete as the resistance to 25-hydroxycholesterol.

In mammalian cells, the addition of sterols enhances the binding of the SCAP/SREBP complex to Insigs, as determined by co-immunoprecipitation (3, 4). To determine whether this can also be demonstrated in Drosophila cells, we transfected the S2 cells with vectors that express Myc-tagged Insig-1 or Insig-2 plus hamster SCAP and HSV-tagged human SREBP-2. When cell extracts were incubated with the anti-Myc antibody, all of the Insigs were precipitated (Fig. 5, top panel). In the presence of Myc-tagged Insig-1, a fraction of SCAP was co-immunoprecipitated with the Insig even in the absence of sterols (lane 7). A similar observation was made in mammalian cells (3, 4). The amount of immunoprecipitated SCAP was increased slightly when the cells were incubated with palmitate plus ethanolamine (lane 8) and to a much greater degree by incubation with sterols (lane 9). In the presence of Insig-2, only a trace amount of SCAP was immunoprecipitated in the absence of sterols (lane 10) and there was no increase with palmitate plus ethanolamine (lane 11). Sterols gave a marked enhancement of SCAP co-immunoprecipitation with Insig-2 (lane 12). Sterols also caused a major increase in the co-immunoprecipitation of the HSV-tagged SREBP-2 (panel labeled SREBP-2 pellet, lanes 9 and 12), but this did not occur in the absence of SCAP (lanes 5 and 6). The latter finding indicates that sterols were enhancing the binding of the SCAP/SREBP-2 to Insigs in Drosophila cells, as they do in mammalian cells.

To determine whether sterols block the movement of mammalian SCAP from ER to Golgi in Drosophila cells, we determined whether the N-linked carbohydrates on SCAP had been converted to a form that is resistant to digestion with endoglycosidase H (endo H), an enzyme that removes only the high-mannose carbohydrates that are present in the ER form of SCAP (Fig. 6). Drosophila cells were transfected with mammalian Insig-1, SCAP, and SREBP-2, and one aliquot of the membrane fraction was immunoblotted to confirm that sterols caused a diminution in the cleavage of SREBP-2 as revealed by a decline in the amount of the intermediate fragment (Fig. 6A). As observed previously, palmitate plus ethanolamine also decreased the cleavage of the mammalian SREBP-2. Other aliquots of the same membrane fraction were digested with trypsin, which generates a trypsin-resistant luminal fragment of SCAP that contains two asparagine-linked carbohydrate chains (6). When this fragment is treated with endo H, three bands are generated. The upper band retains two carbohydrate chains and is fully endo H-resistant, indicating that SCAP has moved to the Golgi and undergone processing by Golgi mannosidases. The middle and lower bands contain either one or two endo H-sensitive chains, which represent the ER form (6). In the absence of Insig, either one or two bands became resistant to endo-H cleavage, indicating that SCAP had visited the Golgi. Sterols had no effect on this pattern (Fig. 6B, lanes 7–9). When Insig-1 was co-expressed, there was a slight reduction in the endo H-resistant carbohydrates, even in the absence of sterols (lanes 7 versus 10). When palmitate plus ethanolamine were added, there was a detectable increase in the number of molecules containing two endo H-sensitive chains (lowest band in lane 11). This increase was nearly complete in the presence of sterols (lane 12), indicating that sterols nearly completely prevented movement of SCAP to the Golgi.

**DISCUSSION**

The current experiments use Drosophila cells to establish that SCAP and Insig-1 or Insig-2 are the minimal requirements necessary in order to achieve sterol-regulated transport of mammalian SREBPs from ER to Golgi. In addition to the mammalian proteins, Drosophila Sec proteins are almost certain to participate in the transport process. In mammalian cells, SCAP moves from ER to Golgi in COPII coated vesicles whose formation requires Sec23/24 and other Sec proteins. In vitro studies showed that mammalian SCAP binds yeast Sec23/24 proteins (18), which are known to facilitate the incorporation of cargo proteins into COPII coated vesicles (19). When membranes are isolated from sterol-treated mammalian cells, SCAP no longer binds the Sec23/24 complex, and this likely accounts for the failure of SCAP to enter COPII vesicles after sterol treatment (18). If mammalian SCAP can interact with yeast Sec23/24, it likely can interact with insect Sec23/24, thus explaining its ability to carry mammalian SCAP to the Golgi in insect cells.

We do not know whether Drosophila proteins are required in order for mammalian Insigs to block the movement of SCAP in the presence of sterols. This block is mediated by the sterol-induced binding of SCAP to Insigs. It is possible that the sterol-induced SCAP/Insig interaction is sufficient to prevent Sec23/24 binding, perhaps by altering the conformation or state of oligomerization of SCAP. Alternatively, the SCAP/Insig complex may bind to insect ER proteins that trap the complex.
Detailed computer searches have failed to reveal a Drosophila protein with significant resemblance to mammalian Insigs. If mammalian Insigs must bind to an ER protein in order to act, then the insect version of such a protein must be able to recognize mammalian Insigs even though no insect Insigs apparently exist.

In the absence of mammalian SCAP, mammalian SREBP-2 is not processed proteolytically in insect cells (Fig. 1B). This deficiency most likely reflects an inability of mammalian SREBP-2 to bind to Drosophila SCAP. Studies in mutant hamster cells (2) and gene-ablated mouse liver (20) have shown an absolute requirement for SCAP to transport SREBPs to the Golgi. In previous studies we showed that Drosophila cells have apparent orthologues of S1P and S2P, which process Drosophila SREBP to its fully cleaved nuclear form (8). In contrast, in the current studies mammalian SREBP-2 was processed only by S1P, but not S2P. Apparently, Drosophila S2P does not recognize mammalian SREBP-2. Our attempts to correct this deficiency by expression of epitope-tagged mammalian S2P in Drosophila cells failed because we were unable to achieve sufficient levels of expression (data not shown).

It is striking that Drosophila SREBP processing is unaffected by sterols, even in cells that express mammalian Insig-1 (Fig. 1A). The sterols must have gained access to the insect ER, as evidenced by the fact that they blocked processing of mammalian SREBP-2 in the transfected Drosophila cells (Fig. 1B). In animal cells, sterols have been shown to alter the conformation of SCAP, as indicated by a change in its pattern of tryptic digestion (21). Sterols may fail to regulate SREBP to its fully cleaved nuclear form (8). In mammalian cells (2) and gene-ablated mouse liver (20) have shown an absolute requirement for SCAP to transport SREBPs to the Golgi. In previous studies we showed that Drosophila cells have apparent orthologues of S1P and S2P, which process Drosophila SREBP to its fully cleaved nuclear form (8). In contrast, in the current studies mammalian SREBP-2 was processed only by S1P, but not S2P. Apparently, Drosophila S2P does not recognize mammalian SREBP-2. Our attempts to correct this deficiency by expression of epitope-tagged mammalian S2P in Drosophila cells failed because we were unable to achieve sufficient levels of expression (data not shown).

It is striking that Drosophila SREBP processing is unaffected by sterols, even in cells that express mammalian Insig-1 (Fig. 1A). The sterols must have gained access to the insect ER, as evidenced by the fact that they blocked processing of mammalian SREBP-2 in the transfected Drosophila cells (Fig. 1B). In animal cells, sterols have been shown to alter the conformation of SCAP, as indicated by a change in its pattern of tryptic digestion (21). Sterols may fail to regulate Drosophila SCAP because they fail to alter its conformation. Alternatively, sterols may alter the conformation of Drosophila SCAP, but the absence of a Drosophila Insig precludes the sterol-induced retention of Drosophila SCAP in the ER. If this latter explanation is correct, it indicates that in the presence of sterols Drosophila SCAP is unable to interact with mammalian Insigs, thus explaining the results in Fig. 1A.

Although insect SCAP does not respond to sterols, it does respond to the mixture of palmitate plus ethanolamine as revealed by the ability of this mixture to block cleavage of Drosophila SREBP. Previous studies with enzyme inhibitors and RNA interference strongly suggest that palmitate plus ethanolamine is converted to phosphatidyethanolamine (PE), which is the active inhibitor (9). Inasmuch as Drosophila cells lack recognizable Insigs, the data suggest that PE can block movement of Drosophila SCAP to the Golgi even in the absence of Insigs. On the other hand, PE did not block the movement of mammalian SCAP unless Insig was co-transfected (Fig. 1B), and even then the block was only partial. These data suggest that Drosophila SCAP is configured differently than mammalian SCAP in that it can respond profoundly to PE, but not sterols. Whether this response reflects direct binding of PE to SCAP, or whether it reflects a change in membrane physical properties is unknown. We have been unable to show that PE regulates SCAP movement in mammalian cells, in part because palmitate is toxic to mammalian cells, because of its conversion to apoptosis-inducing ceramide (22).

From an evolutionary perspective, it appears that the acquisition of an Insig gene may have provided animals with the capacity for feedback regulation of cholesterol synthesis through sterol-regulated exit of the SCAP/SREBP complex from the ER. The ability to reconstitute sterol-regulated transport of SCAP in insect cells provides a new tool that may shed light on the molecular basis by which mammalian cells control the lipid composition and physical properties of their membranes.
8. Seegmiller, A. C., Dobrosotskaya, I., Goldstein, J. L., Ho, Y. K., Brown, M. S., and Rawson, R. B. (2002) *Dev. Cell* **2**, 229–238
9. Dobrosotskaya, I., Seegmiller, A. C., Brown, M. S., Goldstein, J. L., and Rawson, R. B. (2002) *Science* **296**, 879–883
10. Clark, A. J., and Bloch, K. (1959) *J. Biol. Chem.* **234**, 2578–2588
11. Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002) *J. Clin. Invest.* **109**, 1125–1131
12. Sakai, J., Nohturfft, A., Cheng, D., Ho, Y. K., Brown, M. S., and Goldstein, J. L. (1997) *J. Biol. Chem.* **272**, 20213–20221
13. Hua, X., Sakai, J., Brown, M. S., and Goldstein, J. L. (1996) *J. Biol. Chem.* **271**, 10379–10384
14. Sakai, J., Nohturfft, A., Goldstein, J. L., and Brown, M. S. (1998) *J. Biol. Chem.* **273**, 5785–5793
15. Hannah, V. C., Ou, J., Luong, A., Goldstein, J. L., and Brown, M. S. (2001) *J. Biol. Chem.* **276**, 4365–4372
16. Duncan, E. A., Brown, M. S., Goldstein, J. L., and Sakai, J. (1997) *J. Biol. Chem.* **272**, 12778–12785
17. Wang, X., Sato, R., Brown, M. S., Hua, X., and Goldstein, J. L. (1994) *Cell* **77**, 53–62
18. Espenshade, P. J., Li, W.-P., and Yabe, D. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 11684–11689
19. Antonny, B., and Schekman, R. (2001) *Curr. Opin. Cell Biol.* **13**, 438–443
20. Matsuda, M., Korn, B. S., Hammer, R. E., Moon, Y.-A., Komuro, R., Horton, J. D., Goldstein, J. L., Brown, M. S., and Shimomura, I. (2001) *Genes Dev.* **15**, 1206–1216
21. Brown, A. J., Sun, L., Feramisco, J. D., Brown, M. S., and Goldstein, J. L. (2002) *Mol. Cell* **10**, 237–245
22. Mathias, S., Pena, L. A., and Kolesnick, R. N. (1998) *Biochem. J.* **335**, 465–480