

# Sterols block binding of COPII proteins to SCAP, thereby controlling SCAP sorting in ER

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**Sterols inhibit their own synthesis in mammalian cells by blocking the vesicular endoplasmic reticulum-to-Golgi transport of sterol regulatory element-binding protein (SREBP) cleavage-activating protein (SCAP), a sterol-sensing protein that escorts SREBPs. Unable to reach the Golgi, SREBPs are not processed by Golgi-resident proteases, and they fail to activate genes required for cholesterol synthesis. The current studies were designed to reveal whether sterols block SCAP movement by inhibiting synthesis of special vesicles dedicated to SCAP, or whether sterols block SCAP incorporation into common coat protein (COP)II-coated vesicles. Through immunoisolation, we show that SCAP-containing vesicles, formed *in vitro*, also contain vesicular stomatitis virus glycoprotein (VSVG) protein, a classic marker of COPII-coated vesicles. Sterols selectively block incorporation of SCAP into these vesicles without blocking incorporation of VSVG protein. We show that the mammalian vesicular budding reaction can be reconstituted by recombinant yeast COPII proteins that support incorporation of SCAP as well as VSVG into vesicles. Sterols block SCAP incorporation into vesicles by blocking Sar1-dependent binding of the COPII proteins Sec 23/24 to SCAP. These studies demonstrate feedback control of a biosynthetic pathway by the regulated binding of COPII proteins to an endoplasmic reticulum-to-Golgi transport protein.**

Cholesterol homeostasis in mammalian cells is regulated by a feedback mechanism that monitors the level of cholesterol in membranes and alters transcription of genes required for cholesterol supply. Expression of genes required for the uptake and synthesis of cholesterol is controlled by a family of membrane-bound transcription factors called sterol regulatory element-binding proteins (SREBPs) (1). Newly synthesized SREBPs form a complex in the endoplasmic reticulum (ER) with the polytopic membrane protein, SREBP cleavage-activating protein (SCAP). When cells are depleted of sterols, SCAP escorts SREBP from the ER to the Golgi where SREBP undergoes two sequential cleavage events. This proteolysis releases the NH<sub>2</sub>-terminal transcription factor domain of SREBP from the membrane, allowing it to enter the nucleus and activate transcription of genes required for the uptake and synthesis of cholesterol, such as the low-density lipoprotein receptor and 3-hydroxy-3-methylglutaryl CoA reductase (1). When cholesterol accumulates, SCAP is retained in the ER, cleavage of SREBP is blocked, and transcription of target genes declines. This is the first example, to our knowledge, of metabolically regulated transport of a protein from the ER to the Golgi, and the mechanism is unknown. It is not yet known whether sterols block the formation of a special class of vesicles devoted to SCAP or the incorporation of SCAP into a general class of vesicles involved in ER-to-Golgi transport.

In mammalian cells, all previously described secretory proteins exit the ER in vesicles coated with a protein complex called coat protein (COP)II. Vesicles quickly shed their COPII coat and fuse to form the ER–Golgi intermediate compartment (ERGIC), also known as vesicular tubular clusters (2). ERGIC transport complexes move along microtubules and deliver cargo to the Golgi (3). Recently, it has been shown in yeast that glycosylphosphatidylinositol-anchored proteins enter a distinct

class of vesicles at the ER (4). This observation suggests that two classes of vesicles may also form in the ER of mammalian cells and raises the possibility that SCAP may exit the ER in unique vesicles whose formation is inhibited by sterols.

In the current studies, we use immunoisolation and immunocytochemistry to show that SCAP exits the ER in vesicles that contain vesicular stomatitis virus glycoprotein (VSVG), a classic marker of COPII-coated vesicles. For this purpose, we designed an ER vesicle-formation assay that reconstitutes sterol-regulated cargo sorting by using mammalian ER microsomes and purified yeast COPII proteins. In biochemical experiments using purified mammalian COPII proteins, we show that SCAP interacts with COPII proteins involved in cargo selection, and we demonstrate that sterols inhibit this interaction. These data provide a mechanistic explanation for sterol-mediated regulation of ER-to-Golgi transport.

## Materials and Methods

**Cell Culture.** CHO/pGFP-SCAP cells, a stable derivative of SCAP-deficient Chinese hamster ovary (CHO) cells expressing green fluorescent protein (GFP)-SCAP (5), were grown in monolayer at 37°C in an atmosphere of 8–9% CO<sub>2</sub> in medium A (1:1 mixture of Ham's F-12 and DMEM containing 100 units/ml of penicillin and 100 μg/ml of streptomycin sulfate) supplemented with 5% newborn calf lipoprotein-deficient serum. Lipoprotein-deficient serum (density > 1.25 g/ml) was prepared by ultracentrifugation (6). CHO/VSVG-T7 cells, a stable derivative of CHO-K1 cells expressing temperature-sensitive VSVG-T7 (tsO45) (5), were maintained in medium A supplemented with 5% FCS and 0.5 mg/ml of G418. Other materials have been described (5).

**Antibodies.** Polyclonal anti-SCAP IgG-R139 (5), polyclonal anti-SREBP-2 IgG-J911 (7), and control monoclonal antibody IgG-2001 against an irrelevant antigen (*Haemophilus influenzae* type B) (8) have been described. We obtained anti-T7-Tag and anti-T7-Tag horseradish peroxidase conjugate from Novagen and anti-GS28 from StressGen Biotechnologies, Victoria, Canada. Anti-ribophorin I antibody was a generous gift from T. Rapoport, Harvard Medical School.

**In Vitro Vesicle-Formation Assay.** Vesicle reactions using cytosol were performed at 28°C for 15 min as described previously (5). To prepare urea-washed microsomes, microsomes were incubated at 4°C for 30 min in buffer containing 2.5 M urea and then washed in reaction buffer. For yeast COPII experiments, reactions contained microsomes (1 mg/ml), 0.5 mM guanidyl imi-

Abbreviations: 25-HC, 25-hydroxycholesterol; COP, coat protein; DNP, dinitrophenol; ER, endoplasmic reticulum; ERGIC, ER–Golgi intermediate compartment; GFP, green fluorescent protein; GST, glutathione S-transferase; HPCD, hydroxypropyl β-cyclodextrin; SCAP, SREBP cleavage activating protein; SREBP, sterol regulatory element-binding protein; VSVG, vesicular stomatitis virus glycoprotein; CHO, Chinese hamster ovary.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY082671).

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dodiphosphate, 1.5 mM ATP, an ATP regeneration mix, 25  $\mu\text{g}/\text{ml}$  of Sar1p, 15  $\mu\text{g}/\text{ml}$  of Sec23 complex, and 20  $\mu\text{g}/\text{ml}$  of Sec13 complex. Reactions were incubated for 30 min at 25°C. Yeast COPII proteins were prepared as previously described (9).

**Vesicle Immunoprecipitation.** *In vitro* synthesized vesicles were purified by using a modified version of an established protocol (10). Anti-mouse Dynabeads (Dyna) (2–5 mg) coated with monoclonal antibody (IgG-2001 or anti-T7-Tag) were incubated with vesicles from two to four reactions at 4°C. Unbound vesicles were removed from the sample and collected by centrifugation. Bound and unbound vesicles were solubilized in SDS and subjected to SDS/PAGE followed by immunoblotting.

**Immunoelectron Microscopy.** Cultured cells were fixed with 3% (wt/vol) paraformaldehyde–0.1% (wt/vol) glutaraldehyde at 37°C, embedded in agarose, and processed for frozen ultrathin section as described (11). To quantify labeling for SREBP-2, coded samples were examined blindly in the electron microscope. Twenty Golgi stacks were identified at random and scored for gold particles. SREBP-2 labeling in the +sterol sample was corrected for labeling differences between the two samples by using the control anti-GS28 as a standard. Vesicles bound to magnetic beads were fixed as described above. After fixation, vesicles were permeabilized with 0.01% saponin and incubated with rabbit IgG-R139. After washes, vesicles were incubated with goat anti-rabbit IgG conjugated to dinitrophenol (DNP), washed, and embedded in Epon. Sectioning of samples and detection of DNP were carried out by immunogold labeling using anti-DNP mouse monoclonal antibody and gold-conjugated rabbit anti-mouse antibody as previously described (12). Immunogold labeling of VSVG vesicles was quantified directly in the electron microscope.

**Expression and Purification of Recombinant Proteins.** Bacterial expression plasmids were constructed by inserting cDNAs encoding Chinese hamster Sar1a into pGEX-4T-1 (Amersham Pharmacia Biotech). Recombinant glutathione *S*-transferase (GST)–Sar1a proteins were expressed in bacteria and purified by using glutathione agarose (Amersham Pharmacia Biotech) by standard methods. GST was removed proteolytically by treatment with thrombin to generate Sar1a and Sar1a (T39N). cDNA clones for wild-type Sar1a and mutant Sar1a (H79G and T39N) were kind gifts of W. Balch (The Scripps Research Institute) and J. Lippincott-Schwartz (National Institutes of Health), respectively.

mSec23A/hSec24C complex was produced in Sf9 cells by using a baculovirus expression system. Recombinant baculoviruses encoding mouse Sec23A (GenBank accession no. AY082671) and human Sec24C were generated by using the plasmids pFLAG-mSec23A and pHis-hSec24C and the Bac-to-Bac Baculovirus Expression System (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Recombinant 6xHis-tagged hSec24C protein was affinity purified by using Ni-agarose (Qiagen, Chatsworth, CA). After washing, purified proteins were eluted from the resin by using 50 mM Hepes-KOH, pH 7.2/125 mM KOAc/1 mM Mg(OAc)<sub>2</sub>/0.25 M imidazole (13).

**Prebudding Complex Isolation.** COPII prebudding complexes were isolated as reported with modifications (published as supporting information on the PNAS web site, www.pnas.org) (13).

## Results

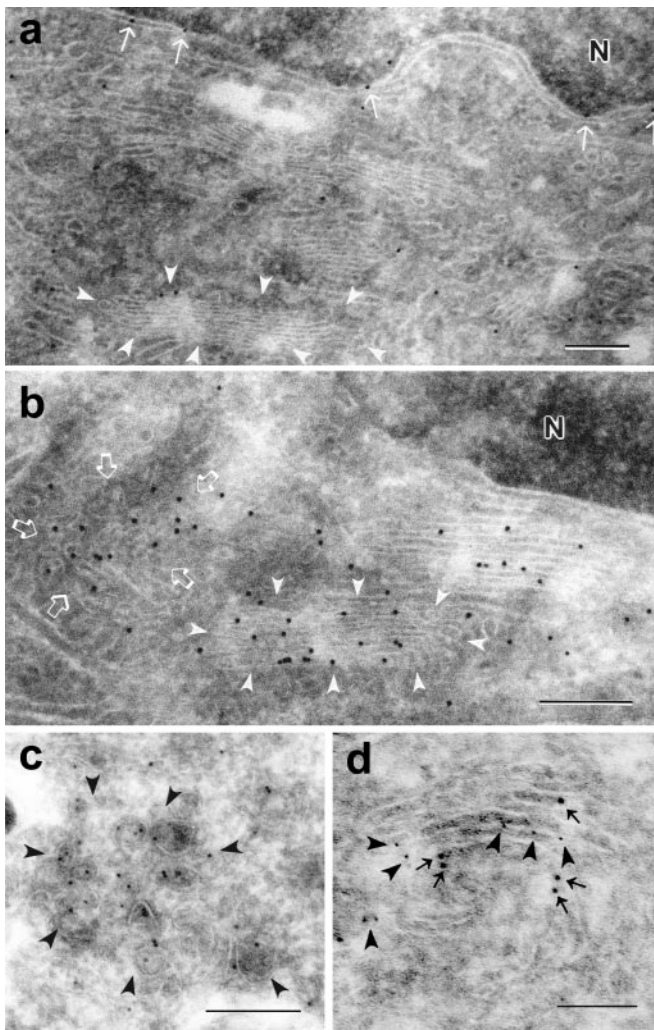
Previous histologic studies have shown at the resolution of light microscopy that SCAP moves from ER to Golgi in response to sterol deprivation. These studies used CHO/pGFP-SCAP cells, a line of SCAP-deficient CHO cells that stably expresses a fusion of GFP to the NH<sub>2</sub> terminus of SCAP (5). To examine the

intracellular localization of GFP-SCAP in more detail, we performed immunoelectron microscopy studies. CHO/pGFP-SCAP cells were cultured in medium containing hydroxypropyl  $\beta$ -cyclodextrin (HPCD) in the presence or absence of sterols. HPCD binds sterols and can be used to deplete cholesterol from intact cells (14). After fixation, cells were processed for immunoelectron microscopy by using antibodies to SCAP, which were visualized with gold-conjugated Protein A. In the presence of sterols, immunogold labeling for GFP-SCAP was present in the ER and nuclear envelope and absent from the Golgi (Fig. 1*a*). Sterol depletion of CHO/pGFP-SCAP cells resulted in dense immunogold labeling of the Golgi apparatus in addition to the ER (Fig. 1*b*). In the absence of sterols, GFP-SCAP was also observed in clusters of vesicles and tubules (open arrowheads, Fig. 1*b*). Immunogold labeling for GFP-SCAP was specific inasmuch as SCAP-deficient cells showed no significant labeling (unpublished data).

In mammalian cells, nascent secretory proteins exit the ER in transport vesicles and pass through the ERGIC en route to the Golgi (3). Protein transport from the intermediate compartment to the Golgi is blocked when cells are incubated at 15°C and proteins accumulate in the ERGIC (15). To test whether SCAP travels to the Golgi through the ERGIC, we examined the localization of GFP-SCAP in cells cultured at 15°C. CHO/pGFP-SCAP cells were depleted of sterols by using HPCD, which initiates GFP-SCAP transport to the Golgi. After 2 h at 37°C, cells were shifted to 15°C for an additional 3 h before fixation. Under these conditions, GFP-SCAP accumulated in clusters of vesicles and tubules characteristic of the ERGIC (Fig. 1*c*). Light microscopy studies verified these results (see Fig. 6, which is published as supporting information on the PNAS web site). Together, these data indicate that SCAP travels to the Golgi via the ERGIC in response to sterol depletion.

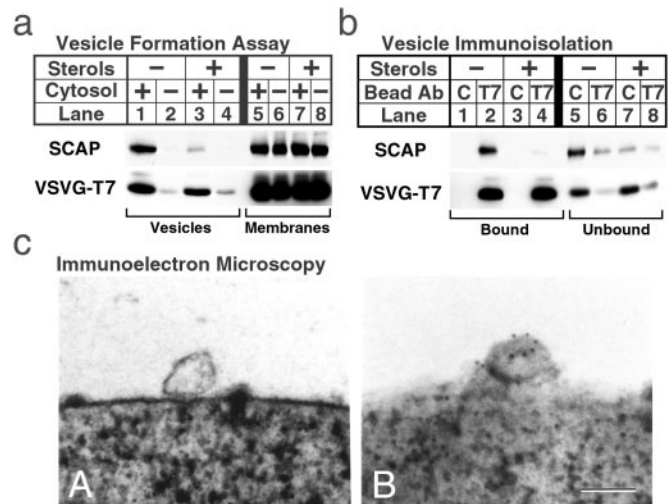
To confirm that SREBP also moves to the Golgi, we examined the localization of SREBP-2 in CHO/pGFP-SCAP cells grown in the presence and absence of sterols by using immunoelectron microscopy. Previous experiments demonstrated that SREBP-2 cleavage is regulated normally by sterols in CHO/pGFP-SCAP cells (5). CHO/pGFP-SCAP cells were cultured in the absence or presence of sterols, fixed, and prepared for electron microscopy. Samples were coincubated with polyclonal antibodies to the NH<sub>2</sub> terminus of SREBP-2 and monoclonal antibodies to the *cis*-Golgi marker GS28. Immunogold labeling in the Golgi for SREBP-2 was quantified in the absence and presence of sterols. In the absence of sterols, SREBP-2 localized to the *cis*-Golgi cisternae (Fig. 1*d*, arrows). Labeling for SREBP-2 in the Golgi decreased more than 6-fold on addition of sterols (Table 1, which is published as supporting information on the PNAS web site). As expected, immunogold labeling for SREBP-2 was observed in the ER and nuclear envelope both in the absence and presence of sterols (unpublished data). Collectively, these data demonstrate that SCAP and SREBP move from the ER to the Golgi in response to sterol depletion.

To explore ER exit of SCAP at a biochemical level, we characterized SCAP vesicles produced in an *in vitro* ER vesicle-formation assay. For these experiments, we used CHO/VSVG-T7 cells, a cloned line of CHO-K1 cells that stably expresses a temperature-sensitive mutant of VSVG (tsO45) with a cytoplasmic, COOH-terminal T7 epitope tag (VSVG-T7). Incubation of CHO/VSVG-T7 cells at 40°C results in accumulation of VSVG-T7 in the ER. VSVG-T7 is incorporated into budding vesicles when the temperature is lowered to 28°C (5). Fig. 2*a* shows an experiment in which CHO/VSVG-T7 cells were incubated at 40°C in the absence or presence of sterols. Microsomes were isolated from cells, and *in vitro* vesicle-formation assays were performed at 28°C to allow exit of VSVG-T7 from the ER. When membranes from cells depleted of sterols were used, SCAP and VSVG-T7 efficiently entered vesicles (Fig. 2*a*,



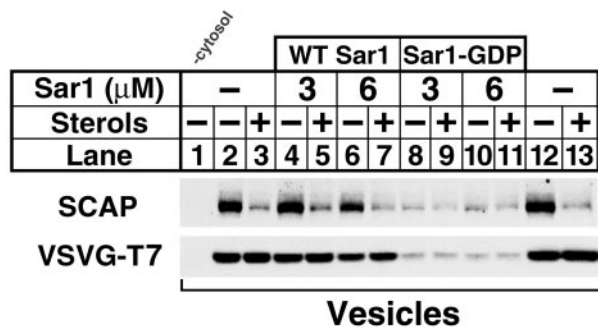
**Fig. 1.** Sterols block transport of SCAP and SREBP-2 to Golgi cisternae. (a and b) CHO/pGFP-SCAP cells were set up as described in *Materials and Methods*. On day 1, cells were switched to medium A supplemented with 5% lipoprotein-deficient serum and 1% HPCD in the presence (a) or absence (b) of sterols (10  $\mu$ g/ml of 25-hydroxycholesterol (25-HC) and 10  $\mu$ g/ml of cholesterol in 0.2% ethanol). After 5 hr at 37°C, cells were fixed and processed for immunoelectron microscopy. Cells were stained with anti-SCAP antibodies followed by incubation with Protein A-gold (10 nm). Arrows in a highlight labeling of the nuclear envelope. Open arrows in b indicate a cluster of vesicles and tubules. Closed arrowheads in both panels identify Golgi stacks. N denotes the nucleus. (Bar = 0.2  $\mu$ m.) (c) CHO/pGFP-SCAP cells were set up in medium A containing 5% FCS. On day 2, cells were switched to medium A supplemented with 5% lipoprotein-deficient serum and 1% HPCD in the absence of sterols. After 2 hr at 37°C, cells were incubated for an additional 3 hr at 15°C. Cells were fixed, processed for immunoelectron microscopy, and stained with anti-SCAP antibodies followed by incubation with Protein A-gold (10 nm). Arrowheads indicate the ERGIC. (Bar = 0.2  $\mu$ m.) (d) CHO/pGFP-SCAP cells were cultured in the absence of sterols for 3 hr at 37°C and processed for immunoelectron microscopy as described in a. Cells were incubated with rabbit IgG-J911 to the NH<sub>2</sub> terminus of hamster SREBP-2 and monoclonal anti-G528, a *cis*-Golgi marker. Primary antibodies were detected using anti-rabbit and anti-mouse gold-conjugated secondary antibodies for SREBP-2 (10 nm) and G528 (5 nm), respectively. Arrows indicate labeling for SREBP-2 and arrowheads highlight labeling for *cis*-Golgi marker, G528. (Bar = 0.1  $\mu$ m.)

lane 1). When membranes were obtained from cells cultured in the presence of sterols, budding of SCAP was inhibited, but budding of VSVG-T7 was unaffected (Fig. 2a, lane 3). Entry of both SCAP and VSVG-T7 into vesicles required addition of cytosol (Fig. 2a, lanes 2 and 4). These data indicate that sterols regulate entry of SCAP, but not VSVG-T7, into ER vesicles (5).



**Fig. 2.** Sterols regulate SCAP entry into VSVG-containing vesicles *in vitro*. (a) On day 0, CHO/VSVG-T7 cells were set up at a density of  $8 \times 10^5$  cells per 10-cm dish in medium A containing 5% FCS and cultured at 40°C. On day 2, cells were switched to medium A supplemented with 5% lipoprotein-deficient serum/50  $\mu$ M compactin/50  $\mu$ M sodium mevalonate/0.5% ethanol/1% HPCD and incubated at 40°C for 1 hr. Cells were washed with PBS and incubated at 40°C for an additional 4 hr in medium A supplemented with 5% lipoprotein-deficient serum/50  $\mu$ M compactin/50  $\mu$ M sodium mevalonate/0.5% ethanol in the absence or presence of 25-HC (1  $\mu$ g/ml). Microsomes were prepared and used in an *in vitro* vesicle-formation assay. The resulting vesicle (lanes 1–4) and membrane (lanes 5–8) fractions were immunoblotted with anti-SCAP and anti-T7-Tag antibodies. Membranes represent 36% of total vesicle fraction. (b) Microsomes prepared in a from cells grown either in the absence or presence of sterols were used to synthesize vesicles *in vitro*. Vesicles from two reactions were incubated with 5 mg of magnetic Dynabeads coated either with a control, irrelevant monoclonal antibody IgG-2001 (C, lanes 1, 3, 5, and 7) or anti-T7-Tag monoclonal antibody (T7, lanes 2, 4, 6, and 8) for 2 hr at 4°C. Beads were collected and washed using a magnet. Unbound vesicles were isolated by sedimentation at  $100,000 \times g$ . Equal fractions of bound (lanes 1–4) and unbound (lanes 5–8) samples were immunoblotted with anti-SCAP and anti-T7-Tag antibodies. (c) On day 0, CHO/VSVG-T7 cells were set up at a density of  $8 \times 10^5$  cells per 10-cm dish in medium A containing 5% FCS and cultured at 40°C. On day 2, cells were switched to medium A supplemented with 5% lipoprotein-deficient serum/50  $\mu$ M compactin/50  $\mu$ M sodium mevalonate/1% HPCD and incubated at 40°C for 1 hr. Cells were washed with PBS and incubated at 40°C for an additional 4 hr in medium A supplemented with 5% lipoprotein-deficient serum/50  $\mu$ M compactin/50  $\mu$ M sodium mevalonate/0.2% ethanol in the absence or presence of sterols (1  $\mu$ g/ml 25-HC and 10  $\mu$ g/ml cholesterol). Microsomes were prepared and used in an *in vitro* vesicle-formation assay. Dynabeads (2 mg) coated with anti-T7-Tag antibody were incubated with vesicles from five reactions by using microsomes prepared from cells grown either in the presence (A) or absence (B) of sterols. Purified vesicles bound to Dynabeads were fixed, incubated with anti-SCAP antibodies, and processed for electron microscopy by using the DNP method (12). (Bar = 0.1  $\mu$ m.)

Sterols could block ER exit of SCAP by two mechanisms: (i) by inhibiting sorting of SCAP but not VSVG-T7 into a common vesicle; or (ii) by inhibiting the formation of a unique SCAP-containing vesicle. To distinguish between these two possibilities, we immunoprecipitated VSVG-containing vesicles and examined their cargo. ER transport vesicles were allowed to form *in vitro* using microsomes from CHO/VSVG-T7 cells cultured in the absence or presence of sterols as in Fig. 2a. Vesicles were immunoprecipitated by using magnetic beads coupled either to a control, irrelevant monoclonal antibody, or anti-T7-Tag monoclonal antibody. Bound and unbound vesicles were examined for the presence of SCAP and VSVG-T7 by immunoblotting. VSVG-T7 containing vesicles were efficiently recovered using magnetic beads coated with anti-T7-Tag antibody but not with a control antibody (Fig. 2b Lower). In the

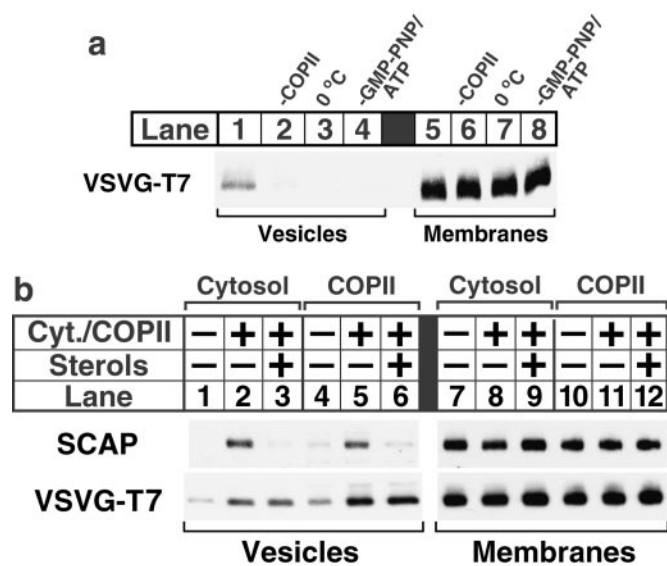


**Fig. 3.** SCAP enters COPII vesicles *in vitro*. CHO/VSVG-T7 cells were cultured in the absence or presence of sterols (1 μg/ml of 25-HC and 10 μg/ml of cholesterol) as in Fig. 2c. Urea-washed microsomes were prepared and used in an *in vitro* vesicle-formation assay. Before addition of cytosol, wild-type hamster Sar1a protein (lanes 4–7) or a GDP-mutant (T39N, lanes 8–11) was added to the indicated final concentration. Resulting vesicle fractions were immunoblotted with anti-SCAP and anti-T7-Tag antibodies.

absence of sterols, SCAP was found in the immunopurified VSVG-T7 vesicles (Fig. 2b, lane 2). Incorporation of SCAP into VSVG-T7 vesicles was blocked by sterols (Fig. 2b, lane 4). No SCAP or VSVG-T7 was found in vesicles recovered using control antibody beads (Fig. 2b, lanes 1 and 3). These data indicate that sterols control sorting of SCAP into a common vesicle used by VSVG.

To test directly whether VSVG-T7 vesicles contained SCAP, we fixed the bead-bound vesicles and examined them by immunoelectron microscopy by using polyclonal anti-SCAP antibody and gold-conjugated secondary antibody. VSVG-T7 vesicles prepared from membranes of cells incubated in the presence of sterols contained few gold particles (Fig. 2cA). Vesicles prepared from membranes of sterol-depleted cells showed strong labeling for SCAP (Fig. 2cB). The morphology of these vesicles (≈80 nm in diameter) was consistent with that previously reported for *in vitro* synthesized ER transport vesicles (10). To quantify the amount of SCAP in these vesicles, we imaged 100 vesicles for each condition (–/+ sterols) and counted the number of gold particles per vesicle (see Fig. 7, which is published as supporting information on the PNAS web site). VSVG-T7 vesicles prepared in the absence of sterols contain 6-fold more gold particles per vesicle ( $4.76 \pm 3.24$ ; mean  $\pm$  SD) than VSVG-T7 vesicles prepared in the presence of sterols ( $0.77 \pm 1.53$ ).

Formation of COPII vesicles is initiated by recruitment of the small GTPase Sar1 to the ER membrane, where it is activated by the exchange of bound GDP for GTP. GTP-bound Sar1 then sequentially recruits two heterodimeric complexes, Sec23/24 and Sec13/31, to the membrane, driving vesicle formation (2). Addition of a GDP-restricted mutant of Sar1, Sar1-GDP, blocks COPII vesicle formation and budding of VSVG *in vitro* (10). To test whether SCAP enters COPII vesicles *in vitro*, we performed *in vitro* vesicle-formation assays by using urea-treated microsomes isolated from CHO/VSVG-T7 cells that had been incubated in the absence and presence of sterols. Reactions were supplemented with either buffer alone, wild-type Sar1, or mutant Sar1-GDP. SCAP efficiently entered vesicles when membranes were obtained from cells incubated in the absence, but not the presence, of sterols (Fig. 3, lanes 2, 3, 12, and 13). Budding of VSVG-T7 was not regulated by sterols. Addition of wild-type Sar1 to 3 or 6 μM had no effect on budding of SCAP or VSVG-T7 (Fig. 3, lanes 4–7), presumably because a saturating level of Sar1 is already present in the cytosol. By contrast, addition of 3 μM Sar1-GDP markedly reduced the amount of SCAP and VSVG-T7 in the vesicle fraction, suggesting



**Fig. 4.** Sterols regulate sorting of SCAP into yeast COPII vesicles. (a) CHO/VSVG-T7 cells were cultured in the absence of sterols as described in Fig. 2c. Microsomes were prepared and used in an *in vitro* vesicle-formation assay. Purified yeast COPII proteins were added to the following final concentrations: Sar1 (25 μg/ml), Sec23 complex (15 μg/ml), and Sec13 complex (20 μg/ml). Resulting vesicle and membrane fractions were immunoblotted with anti-SCAP and anti-T7-Tag antibodies. Membranes represent 6% of total vesicle fraction. (b) CHO/VSVG-T7 cells were cultured in the absence or presence of sterols (1 μg/ml of 25-HC and 10 μg/ml of cholesterol) as in Fig. 2c. Vesicle reactions were performed using either cytosol (lanes 1–3 and 7–9) or yeast COPII proteins (lanes 4–6 and 10–12). Cytosol and yeast COPII reactions contained GTP and GMP-PNP, respectively. Purified yeast COPII proteins were added to the following final concentrations: Sar1 (25 μg/ml), Sec23 complex (15 μg/ml), and Sec13 complex (20 μg/ml). Resulting vesicle (lanes 1–6) and membrane (lanes 7–12) fractions were immunoblotted with anti-SCAP and anti-T7-Tag antibodies. Membranes represent 25 and 5% of total vesicle fraction for cytosol and COPII reactions, respectively. Lanes 4–6 (COPII reactions) contain 3.5 times more sample than lanes 1–3 (cytosol reactions).

that SCAP and VSVG exit the ER in COPII vesicles (Fig. 3, lanes 8–11).

That SCAP entered COPII vesicles in a crude reaction prompted us to examine whether purified COPII proteins were sufficient to reconstitute sterol-regulated budding of SCAP. First, we tested whether purified COPII proteins from *Saccharomyces cerevisiae* could support the formation of vesicles from mammalian microsomes *in vitro*. Microsomes from CHO/VSVG-T7 cells grown in the absence of sterols were used in an *in vitro* vesicle-formation reaction that lacked cytosol, but included yeast COPII proteins, GMP-PNP (a nonhydrolyzable analog of GTP), and an ATP regeneration system. Under these conditions, we detected VSVG-T7 in the vesicle fraction (Fig. 4a, lane 1). Budding of VSVG-T7 required yeast COPII proteins, temperature, and added nucleosides (Fig. 4a, lanes 2–4). Budding of VSVG was more efficient in the presence of GMP-PNP than GTP (unpublished data). These data indicate that yeast COPII proteins can substitute for mammalian COPII proteins in mediating the budding of ER-derived vesicles *in vitro*.

Next, we tested whether mammalian SCAP budding retains sterol sensitivity when vesicle formation is mediated by yeast COPII proteins. CHO/VSVG-T7 cells were grown in the absence or presence of sterols, and isolated microsomes were used for *in vitro* vesicle-formation reactions with either rat liver cytosol or purified yeast COPII proteins. In reactions containing cytosol, SCAP entered vesicles in a sterol-regulated fashion (Fig. 4b, lanes 2 and 3). The same results were obtained when yeast COPII proteins were substituted for rat liver cytosol (Fig. 4b,

lanes 5 and 6). Unlike SCAP, the entry of VSVG-T7 into vesicles was unaffected by sterols (Fig. 4b, lanes 2, 3, 5, and 6). Incorporation of SCAP and VSVG-T7 into yeast COPII vesicles was only one-fifth as efficient as the incorporation when mammalian cytosol was present. To correct for this difference, lanes 4–6 of Fig. 4b contain a 5-fold higher proportion of the total vesicle fraction as compared with lanes 1–3.

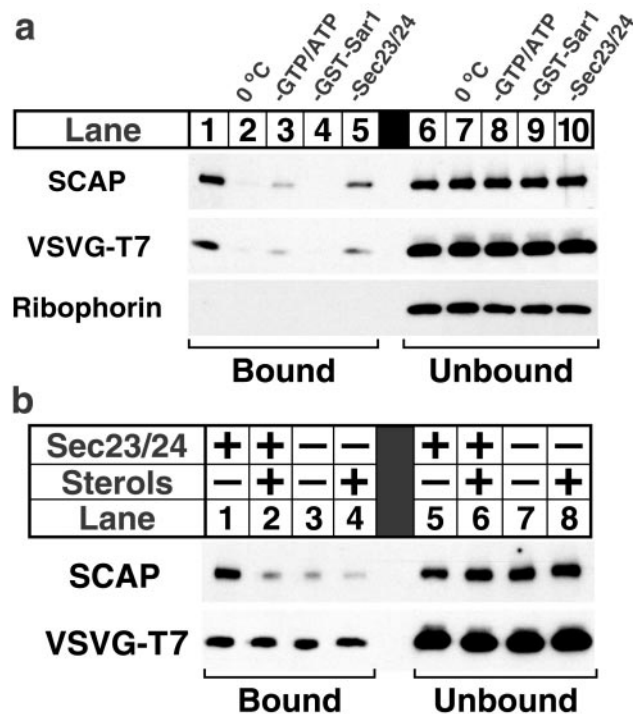
Membrane-bound secretory proteins are incorporated into COPII vesicles via interaction of COPII proteins with cytoplasmic domains of cargo molecules. Complexes between Sar1, Sec23/24, and cargo molecules can be isolated when GTP hydrolysis by Sar1 is inhibited (13, 16). After addition of a GTP-restricted mutant of Sar1 fused to glutathione *S*-transferase (GST-Sar1-GTP), these prebudding complexes are isolated from detergent solubilized membranes using glutathione agarose beads. Mammalian cells contain two genes coding for Sec23 (A and B) and four genes for Sec24 (A–D) (17, 18). We used a baculovirus expression system to produce a complex of Sec23A and Sec24C (see Fig. 8, which is published as supporting information on the PNAS web site), the two isoforms that display the broadest tissue expression (19). Purification of 6xHis-Sec24C on a nickel column resulted in recovery of stoichiometric amounts of Sec23A protein, indicating that the proteins form a 1:1 complex as shown previously (20). In preliminary experiments, we found that purified Sec23/24 bound to ER microsomes only in the presence of Sar1 protein (unpublished data) (13).

Fig. 5a shows an experiment designed to test whether SCAP forms a complex with purified mammalian COPII proteins *in vitro*. Urea-washed microsomes from CHO/VSVG-T7 cells grown in the absence of sterols were incubated with GST-Sar1-GTP and Sec23/24 in the presence of ATP and GTP for 15 min at 28°C. The microsomes were reisolated by centrifugation, and bound proteins were solubilized with digitonin. Proteins that were bound to GST-Sar1-GTP were recovered on glutathione agarose beads. In this experiment, SCAP bound to glutathione-agarose beads in a reaction that required temperature, GTP/ATP, GST-Sar1-GTP, and to some extent Sec23/24 (Fig. 5a Top, lanes 1–5). Likewise, VSVG-T7 was bound under the same conditions (Fig. 5a Middle) (13). Binding of SCAP and VSVG-T7 to GST-Sar1-GTP was specific for vesicle cargo proteins, because the ER resident protein, ribophorin, did not bind in the same experiment (Fig. 5a Bottom).

To test whether sterols regulate binding of COPII proteins to SCAP, we performed the COPII-binding assay by using non-urea-treated microsomes prepared from CHO/VSVG-T7 cells grown in the absence or presence of sterols. In the absence of sterols, SCAP bound to GST-Sar1-GTP in a reaction that required Sec23/24, and binding was blocked by sterols (Fig. 5b, lanes 1–4). Recovery of VSVG-T7 was not affected by sterols and did not require the addition of Sec23/24 (Fig. 5b Lower). These data suggest that residual Sec23/24 associated with membranes binds preferentially to VSVG-T7 and is sufficient to mediate VSVG-T7, but not SCAP, binding to GST-Sar1-GTP. Treatment of membranes with urea increased binding of COPII to SCAP in the presence of sterols (unpublished data), suggesting that urea may disrupt the interaction of SCAP with a protein involved in sterol-mediated ER retention. We conclude that sterols regulate binding of SCAP to Sar1 and Sec23/24.

## Discussion

Sterols control the activation of SREBPs by regulating ER-to-Golgi transport of SCAP. In the presence of sterols, SREBP/SCAP is retained in the ER and is unable to interact with Golgi localized proteases. Here, we demonstrate that sterols inhibit the sorting of SREBP/SCAP into COPII vesicles at the ER, thus preventing transport to the Golgi. Sterol-regulated sorting is specific for SREBP/SCAP, as incorporation of VSVG into



**Fig. 5.** Sterols regulate binding of SCAP to Sec23/24 complex. (a) CHO/VSVG-T7 cells were cultured in the absence of sterols as in Fig. 2c. Urea-washed microsomes (1 mg/ml) were prepared and incubated with GST-Sar1-GTP [GST-Sar1(H79G)] (75  $\mu$ g/ml) and purified mammalian Sec23 complex (31  $\mu$ g/ml) at 28°C for 15 min. After centrifugation, microsomes were solubilized by the addition of digitonin. Detergent-solubilized protein complexes were isolated by using glutathione agarose. Bound (lanes 1–5) and unbound fractions (lanes 6–10) were immunoblotted with anti-SCAP, anti-T7-Tag, and anti-ribophorin I antibodies. Unbound fraction represents 5% of bound fraction. (b) CHO/VSVG-T7 cells were cultured in the absence or presence of sterols (1  $\mu$ g/ml 25-HC and 10  $\mu$ g/ml cholesterol) as in Fig. 2c. Microsomes (1 mg/ml) were prepared and incubated with GST-Sar1-GTP (75  $\mu$ g/ml) and Sec23 complex (37.5  $\mu$ g/ml) at 28°C for 15 min and processed as described in a. Bound (lanes 1–4) and unbound fractions (lanes 5–8) were immunoblotted with anti-SCAP and anti-T7-Tag antibodies. Unbound fraction represents 5% of bound fraction.

COPII vesicles is unaffected by sterols. Considered together with previous data (5), these data provide a mechanism for lipid-controlled protein sorting in the ER.

SREBP/SCAP moves from ER to Golgi by the classical secretory pathway. Evidence for this conclusion comes from four sources. First, SCAP enters COPII vesicles *in vitro* (Figs. 3 and 4b). Second, vesicle immunoisolation experiments show that SCAP exits the ER in transport vesicles that contain VSVG and exhibit normal size and morphology (Fig. 2). Third, GFP-SCAP accumulates in the ERGIC when sterol-depleted cells are cultured at 15°C (Fig. 1c), demonstrating that SREBP/SCAP passes through the ERGIC en route to the Golgi. Lastly, immunocytochemistry experiments show that both GFP-SCAP and SREBP-2 localize to Golgi cisternae when CHO/GFP-SCAP cells are depleted of sterols (Fig. 1 b and d).

The establishment of assays monitoring the earliest events in ER-to-Golgi transport allowed us to examine the role of sterols in the ER exit of SCAP. Three independent lines of evidence demonstrate that sterols act by regulating the sorting of SREBP/SCAP into COPII-coated vesicles. First, immunisolated ER vesicles prepared using microsomes from cells cultured in the presence of sterols contained 6-fold less SCAP than vesicles prepared from sterol-depleted cells (Fig. 2c and Fig. 7). Second,

sterol treatment *in vivo* led to a reduction in the entry of SCAP, but not VSVG, into yeast COPII-coated vesicles *in vitro* (Fig. 4). The ability of purified COPII proteins to support the sterol-regulated budding of SCAP highlights the fact that information regarding the “sterol state” of the cell is due to stable alterations in the ER membrane and not a cytosolic component. Third, cargo molecules are actively sorted into COPII vesicles through the formation of prebudding complexes with the mammalian COPII proteins Sar1 and Sec23/24. SCAP forms a complex with GST-Sar1-GTP that requires addition of Sec23/24 protein, and formation of this complex is regulated by sterols (Fig. 5 *a* and *b*). Collectively, these results demonstrate that sterols regulate the sorting of SCAP into COPII vesicles that contain VSVG, rather than controlling formation of a novel class of ER-derived vesicle that is unique to SREBP/SCAP.

SCAP forms a complex with the COPII proteins Sar1 and Sec23/24 (Fig. 5) and is packaged into COPII vesicles *in vitro*. Currently, the domain of SCAP required for this interaction is unknown. Extensive characterization of ER export signals in transmembrane secretory proteins has failed to identify a general consensus sequence necessary for COPII binding and efficient ER export. The two best-characterized motifs for COPII binding contain a diacidic (DxE) or diphenylalanine sequence located in short cytoplasmic domains at the COOH terminus of membrane proteins, such as VSVG, ERGIC-53, and the p24 family of proteins (21–23). In contrast, the large cytosolic COOH terminus of SCAP forms a complex with SREBP and bears little structural similarity to these proteins. Thus, SCAP may interact directly with COPII through a novel export sequence or indirectly through another protein. In either case, the ability of COPII to recognize sorting signals in SCAP and VSVG has been conserved between yeast and mammals through evolution, because yeast COPII proteins package both SCAP and VSVG specifically into ER vesicles (Fig. 4).

Current data suggest that SCAP functions as the sterol sensor in this system. Two single amino acid changes (Y298C and D443N) in the NH<sub>2</sub>-terminal transmembrane segments of SCAP disrupt sterol sensitivity and cause SCAP to exit the ER in the presence of sterols (24). This sterol-sensing domain of SCAP (transmembrane segments 2–6) is believed to interact with an ER retention protein in the presence of sterols (25). Mutations in the sterol-sensing domain may disrupt binding of SCAP to sterols or the retention protein. Our data support a model in which SCAP binds to a retention protein in the presence of sterols and is unable to interact with COPII proteins. When cells are depleted of sterols, SCAP is released, interacts with COPII proteins, and exits the ER. The inability of SCAP to interact with COPII may be due to a conformational change in SCAP or to sequestration of SCAP in a region of the ER that is inactive for vesicle formation. A more detailed understanding of this ER retention mechanism awaits the identification of the SCAP ER retention protein.

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