Depletion of β-COP reveals a role for COP-I in compartmentalization of secretory compartments and in biosynthetic transport of caveolin-1

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Styers ML, O’Connor AK, Grabski R, Cormet-Boyaka E, Sztul E. Depletion of β-COP reveals a role for COP-I in compartmentalization of secretory compartments and in biosynthetic transport of caveolin-1. Am J Physiol Cell Physiol 294: C1485–C1498, 2008. First published April 2, 2008; doi:10.1152/ajpcell.00010.2008.—We have utilized small interfering RNA (siRNA)-mediated depletion of the β-COP subunit of COP-I to explore COP-I function in organelar compartmentalization and protein traffic. Reduction in β-COP levels causes the colocalization of markers for the endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC), Golgi, trans-Golgi network (TGN), and recycling endosomes in large, globular compartments. The lack of spatial differentiation of these compartments is not due to a general collapse of all cellular organelles since markers for the early endosomes and lysosomes do not redistribute to the common structures. Anterograde trafficking of the transmembrane cargo vesicular stomatitis virus membrane glycoprotein and of a subset of soluble cargoes is arrested within the common globular compartments. Similarly, recycling traffic of transferrin through the common compartment is perturbed. Furthermore, the trafficking of caveolin-1 (Cav1), a structural protein of caveolae, is arrested within the globular structures. Importantly, Cav1 coprecipitates with the γ-subunit of COP-I, suggesting that Cav1 is a COP-I cargo. Our findings suggest that COP-I is required for the compartmentalization of the ERGIC, Golgi, TGN, and recycling endosomes and that COP-I plays a novel role in the biosynthetic transport of Cav1.

Caveolin-1 (Cav1); trans-Golgi network; endoplasmic reticulum-Golgi intermediate compartment; recycling endosome; coatamer

THE SECRETORY AND ENDOSONAL PATHWAYS consist of distinct compartments that communicate through vesicular transport (5). Cargo proteins are moved along each pathway by sequential packaging into vesicular carriers that deliver proteins and lipids to the next station along the pathway (64). The packaging of cargo into vesicles requires sorting away from proteins that are residents of the donor compartment and are not to be transported. Cargo packaging appears to be mediated by the assembly of coats on the cytoplasmic aspect of the donor compartment (5). Such coats selectively sequester cargoes by binding to sorting motifs in the cytoplasmic tails of transmembrane proteins and recruiting them into vesicular buds before vesicle scission (38). The COP-I coat represents one of the packaging machineries operational within the early secretory pathway (43).

The COP-I coat assembles by the en bloc transfer of a 700-kDa coatamer complex from the cytoplasm to the membrane (76). Coatamer is composed of seven subunits, which can be divided into two subcomplexes composed of the α-, β′-, and ε-subunits and the γ-, ζ-, δ-, and β-subunits ([20] and Fig. 1A). COP-I coats are recruited primarily to the membranes of the endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC) and the Golgi (24) and also have been detected on the trans-Golgi network (TGN) and on early endosomes (12, 27, 78). COP-I has been shown to regulate retrograde transport of proteins that cycle between the Golgi and the ER (43). This function of COP-I is deduced from studies showing direct interactions between COP-I subunits and the cytoplasmic tails of p24 family members and the requirement for these interactions in retrograde transport of these proteins (4, 21, 69). COP-I has been shown to bind both dityrosine signals and diaromatic motifs in the cytoplasmic tails of these cargo proteins (21).

Although retrograde traffic between the Golgi and the ER is the best-defined role for COP-I, several lines of evidence suggest that COP-I also plays roles in protein traffic between other compartments. COP-I appears essential for the anterograde transport of vesicular stomatitis virus membrane glycoprotein (VSV-G) from the ER to the Golgi since microinjection of function-blocking antibodies against the β-COP subunit of COP-I inhibits anterograde transport of VSV-G protein at the ER-Golgi interface (57, 58). In addition, pharmacological agents and genetic manipulations that block COP-I recruitment to membranes also inhibit anterograde traffic. Specifically, preventing ADP-ribosylation factor (ARF) activation by treating cells with brefeldin A (BFA), a known inhibitor of the Golgi-specific BFA-resistant factor 1 (GBF1) guanine nucleotide exchange factor that activates ARF at the ER-Golgi interface (9, 24), or by expressing dominant negative mutants of GBF1 or ARF1, prevents COP-I recruitment to membranes and inhibits anterograde transport (13, 24). Furthermore, COP-I vesicles may traffic both retrograde and anterograde proteins since isolated COP-I vesicles can be separated into different subclasses, with some containing recycling proteins (KDEL receptor) and some containing anterograde-directed cargoes (proinsulin and VSV-G) (53).

Experimental evidence suggests that COP-I also plays a role in endosomal transport. The ldl-F Chinese hamster ovary (CHO) cell line expresses a mutant form of ε-COP with a temperature-sensitive point mutation that, at the nonpermissive temperature, results in degradation of ε-COP and inhibits COP-I function (29, 30). In these cells, many functions of early endosomes are compromised, including reduced uptake of VSV or Semliki Forest virus, sorting of EGF to lysosomes, and recycling of transferrin (12). Furthermore, function-blocking

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Depletion of β-COP results in fragmentation of the Golgi complex. A: schematic of subunit arrangements in COP-I subcomplex 1 and 2. Known protein-protein interactions are indicated. ARF, ADP-ribosylation factor. B: cells transfected with control or two different β-COP small interfering RNAs (siRNAs) for 48 h were lysed, and the lysates were analyzed by Western blot analysis with indicated antibodies. β-COP levels in depleted cells were reduced to 75–80% of control cells. Levels of other COP-I subunits (α-COP, γ-COP, and ε-COP) were unaffected. Calreticulin is shown as a loading control. C: cells transfected with control or two different β-COP siRNAs for 48 h were fixed and processed for immunofluorescence (IF) with antibodies against β-COP, ε-COP-subunit of COP-I, whereas the α- and γ-subunits form one subcomplex of COP-I, whereas the γ-, ζ-, δ-, and β-subunits form a second subcomplex (see Ref. 20 and Fig. 1A). Although both of these subcomplexes are involved in COP-I binding to ARF, ARF GTPase activating protein 2 (GAP2) and KXXX-containing cargoes, subcomplex I is also involved in binding to Cdc42 and WXXW/Y/F cargoes. This suggests that depletion of the different subunits may have different cellular outcomes. The effects of depleting any subunit of the γ(δβ) subcomplex have not been previously explored. Therefore, to further explore the role of COP-I in cellular traffic, we utilized a strategy of small interfering RNA (siRNA)-mediated depletion of the β-COP subunit of COP-I.

Here, we show that depletion of β-COP has a dramatic effect on the architecture of the secretory pathway and leads to a novel phenotype in which the ERGIC, Golgi, TGN, and recycling endosomal compartments collapse into common, large, globular structures dispersed throughout the cytoplasm. Importantly, the common compartments lack markers for early or late endosomes and lysosomes, indicating that this effect is not due to a general collapse of cellular organelles. Interestingly, the effects differ significantly from compartment fragmentation and collapse observed in cells in which COP-I association with membranes is inhibited by BFA or by expression of dominant negative ARF or GBF1. The appearance of the common globular structures correlates with traffic arrest of VSV-G in these structures and a block in secretory traffic, confirming the critical role of COP-I in anterograde traffic. The common compartments are accessible to endocytosed transferrin, but its recycling to cell surface appears inhibited in β-COP-depleted cells.

We also show that trafficking of caveolin-1 (Cav1), a structural component of caveolae, is inhibited in β-COP-depleted cells. Using a temporary block in protein synthesis, we show that biosynthetic transport of Cav1 is blocked in the fragmented compartments in β-COP-depleted cells or in ldl-F cells at the nonpermissive temperature. Consistent with a role for COP-I in Cav1 traffic, coimmunoprecipitation demonstrated that Cav1 interacts with the γ-subunit of COP-I, a subunit shown previously to bind cargo proteins (4). Together, our observations suggest that the COP-I coat is required for compartmentalization of organelles of the secretory pathway and for the biosynthetic traffic of Cav1.

**MATERIALS AND METHODS**

**Cell culture and transfections.** HeLa cells were grown in MEM with glucose and L-glutamine (Mediatech, Comprehensive Cancer Center of University of Alabama, Birmingham, AL), supplemented with 10% fetal bovine serum.
with 10% FBS (Life Technologies, Grand Island, NY), 1 mM sodium pyruvate, 0.075% sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Mediatech). Ldl-F and Idl-F(LLD-F) cells were a generous gift of Monty Krieger (Massachusetts Institute of Technology, Cambridge, MA) and were cultured as previously described (30).

DNA transfections were performed using TransIT-LT1 polyamine transfection reagents (Mirus, Madison, WI) according to manufacturer protocols. Plasmid-containing mcg-tagged coelchi was generated as described (26). siRNA oligos were transfected with SilenTect reagent (Bio-Rad, Hercules, CA) according to manufacturer protocol. Control siRNA no. 1 and predesigned β-COP siRNAs (ID no. 146742 and ID no. 146741) were from Ambion (Austin, TX) and were transfected at a concentration of 44 nM. Cells were transfected for 48 h before processing.

Antibodies and reagents. Rabbit polyclonal antibodies against GM130 have been described previously (49). Monoclonal anti-giantin G1/133 antibodies (44) and monoclonal anti-ERGIC-53 G1/93 antibodies (65) were a generous gift from Dr. Hans-Peter Hauri (University of Basel, Basel, Switzerland). Sheep polyclonal Golgin-84 antibodies were a generous gift from Martin Lowe (University of Manchester, Manchester, UK) (15). The following commercially available antibodies were used: polyclonal β-COP, α-COP, and calreticulin from Affinity BioReagents (Golden, CO), mouse polyclonal anti-γ-COP (COPG2) and anti-ε-COP (COPE) from Abnova (Taipei City, Taiwan), monoclonal anti-Golgin-97 from Invitrogen (Carlsbad, CA), polyclonal anti-Cav1, monoclonal anti-Cav1, anti-GM130, anti-syntaxin-6, anti-Rab11, and anti-EEA1 from BD Transduction Laboratories (Lexington, KY), polyclonal anti-giantin from Covance (Princeton, NJ), sheep anti-TGN46 from AbD Serotec (Raleigh, NC), monoclonal anti-γ-adaptin (AP-1) from Sigma-Aldrich (St. Louis, MO), monoclonal anti-transferrin receptor from Zymed Laboratories/Invitrogen (Carlsbad, CA), monoclonal anti-LAMP-1 (HA43) and anti-LAMP-2 (HB4) from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA), and monoclonal anti-green fluorescent protein (GFP) and anti-mannose-6-phosphate receptor from Abcam (Cambridge, UK), and polyclonal anti-myct (A-14) from Santa Cruz Biotechnology (Santa Cruz, CA). The following secondary antibodies were used: goat anti-rabbit and anti-mouse and donkey anti-sheep conjugated with Alexa Fluor 488 and Alexa Fluor 594 from Molecular Probes/Invitrogen (Eugene, OR), and horseradish peroxidase-labeled goat anti-rabbit and goat anti-mouse from Zymed. Human transferrin conjugated with Alexa Fluor 594 was from Molecular Probes/Invitrogen. Unconjugated human transferrin was from Sigma-Aldrich.

Immunofluorescence microscopy. For immunofluorescence (IF), unless otherwise noted, cells were fixed in 3% paraformaldehyde in PBS for 15 min at room temperature. Paraformaldehyde was quenched with 10 mM ammonium chloride, and cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Cells stained for surface Cav1 were fixed with ice-cold methanol for 5 min and were not further permeabilized. The coverslips were washed (3 times, 2 min per wash) with PBS, then blocked in 0.4% fish skin gelatin in PBS for 5 min, followed by blocking in 2.5% goat serum in PBS for 5 min. Cells were incubated with primary antibody diluted in 0.4% fish skin gelatin in PBS for 45 min at 37°C. Coverslips were washed (5 ×, 5 min per wash) with PBS containing 0.2% Tween 20. Secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 were diluted in 2.5% goat serum in PBS and incubated on coverslips for 30 min at 37°C. Coverslips were washed as described above and mounted on slides in 91:1 glycerol/PBS with 0.1% q-phenylenediamine Fluorescence was visualized with a Leitz Orthoplan epifluorescence microscope (Wetzlar, Germany). Optical sections were captured with a CCD high-resolution camera equipped with a camera/computer interface. Images were analyzed with IPLab Spectrum software (Scanalytics, Fairfax, VA). Images were quantified with National Institutes of Health Image J Software using either the calculated mean intensity measurement or the JACoP colocalization plugin. Identical thresholds were applied to all images, and at least 5–10 images were quantified per condition. Colocalization data is presented using the overlap coefficient calculated by the software. Significance was assessed using the Student’s t-test. Scale bars are 10 μm.

SDS-PAGE, immunoblotting, immunoprecipitations, and sucrose gradient fractionation. For Western blot analyses, HeLa cells were solubilized in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) supplemented with complete protease inhibitors (Roche Diagnostics, Indianapolis, IN) 48 h after transfection. After incubation at 4°C for 30 min, samples were centrifuged at 16,000 g for 20 min. Protein concentration was assessed using the bicinchoninic acid (BCA) protein assay from Pierce (Rockford, IL). Equal amounts of soluble lysates were subjected to SDS-PAGE and immunoblotted as previously described (23). Bound antibodies were detected using SuperSignal West Pico chemiluminescence substrate from Pierce. Western blot analyses were quantified from at least three independent experiments using Labworks software from UVP (Upland, CA).

For coimmunoprecipitations, HeLa cells were solubilized in buffer A (10 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EGTA, and 1.0 mM MgCl2) containing 1% Triton X-100 and complete protease inhibitors. After incubation at 4°C for 30 min, samples were centrifuged at 16,000 g for 20 min. Protein concentration was assessed using the Bio-Rad protein assay. Equal amounts of soluble lysate were incubated at 4°C with 2 μg of the appropriate antibody for 16 h at 4°C, followed by a 2-h incubation with 20 μL of 50% (vol/vol) protein A or protein G Sepharose 4FF (Amersham Bioscience, Uppsala, Sweden). Beads were recovered by centrifugation and then washed five times with solubilization buffer. Precipitates were analyzed by SDS-PAGE followed by immunoblotting as described above.

Sucrose gradients (5–30%) to examine COP-I complex stability were generated by layering 1.9 ml of the following sucrose solutions made in buffer A: 5, 10, 15, 20, 25, and 30% sucrose. Lysates from HeLa cells transfected with control and β-COP siRNA for 48 h were solubilized and cleared as described above for coimmunoprecipitations. Protein concentration was assessed using the Bio-Rad protein assay. Equal amounts of protein were brought up to an equal volume using solubilization buffer. Lysates were layered on top of the gradients, and gradients were centrifuged at 180,000 g for 24 h in a Beckman SW41 rotor. Fractions (0.7 ml) were taken from the top of the gradient and immediately supplemented with complete protease inhibitors. Fractions were subjected to SDS-PAGE and analyzed by immunoblot as described above.

Lipid raft sucrose gradient fractionation was performed as previously described (7). Briefly, HeLa cells were transfected with control and β-COP siRNA for 48 h and were lysed in MES-buffered saline (MBS) buffer (25 mM MES, 150 mM NaCl, pH 6.5) containing 1% Triton X-100 and complete protease inhibitors for 20 min at 4°C. Cells were homogenized using 10 strokes of a Dounce homogenizer. Equal amounts of protein were brought up to a volume of 1.5 ml using solubilization buffer. Homogenate was then added to 1.5 ml of 80% sucrose in MBS. This layer was overlaid with 6 ml 30% sucrose and 4 ml of 5% sucrose. Gradients were centrifuged at 240,000 g for 18 h at 4°C. Fractions (1 ml) were collected from the top of the gradient and immediately supplemented with complete protease inhibitors. The pellet was resuspended by dounce homogenization in 1 ml MBS containing protease inhibitors and designated as fraction P. Fractions were subjected to SDS-PAGE and analyzed by immunoblot as described above.

Traffic of VSV-G protein. HeLa cells were transfected with control or anti-β-COP siRNA oligos for 24 h. Cells were then transfected with temperature-sensitive ts045 VSV-G-GFP (2, 3, 40, 60, 77) at the nonpermissive temperature and incubated at nonpermissive temperature of 42°C for 24 h. Cells were next shifted to the permissive temperature of 32°C and incubated for 0–12 h. Cells were washed 3× in PBS, fixed with 3% paraformaldehyde, and processed for IF. VSV-G-GFP was visualized using antibodies to GFP.
Total secretion and cochlin secretion. HeLa cells were transfected with control or β-COP siRNAs for 24 h. Cells were then transfected with a plasmid containing myc-tagged cochlin for an additional 24 h (26). Cells were incubated in DMEM lacking cysteine and methionine for 30 min at 37°C. [35S]-labeled cysteine-methionine (100 μCi) was then added to each well, and the cells were incubated at 37°C for 30 min. Cells were then washed twice with media, and prewarmed media was added. At 0 min, 30 min, 1 h, 2 h, and 4 h, the media was removed and cells were washed and lysed as described above for Western blot analyses. Protein concentration was assessed using the BCA protein assay from Pierce. To assay total secretion, equal amounts of soluble lysates and media were subjected to SDS-PAGE, and labeled proteins were detected by autoradiography. To assay cochlin secretion, cochlin immunoprecipitations were performed as described above from both lysates and media, except RIPA buffer containing complete protease inhibitors was used as the solubilization and wash buffer. Immunoprecipitated proteins were resolved by SDS-PAGE, and labeled proteins were detected by autoradiography.

BFA and cycloheximide treatments. HeLa cells were treated with 10 μg/ml of BFA (Sigma-Aldrich) for 30 min at 37°C. Cells were washed 3× with PBS, fixed, and processed for IF.

HeLa cells were transfected with either control or β-COP siRNA oligos for 48 h. Cells were either mock treated or treated with 200 μg/ml cycloheximide (Sigma-Aldrich) for 3 h at 37°C (51). Cells were then washed 3× in PBS, fixed, and processed for IF.

Transferrin endocytosis and uptake. HeLa cells were transfected with either control or β-COP siRNA oligos for 48 h. To assay transferrin endocytosis, cells were first rid of endogenous transferrin by incubating in serum-free DMEM containing 20 mM HEPEs and 0.1% BSA (DMEM + HB). Cells were then incubated in 50 μg/ml human transferrin conjugated to Alexa 594 in DMEM + HB for 1 h at 37°C. Cells were next washed 3× in DMEM + HB. Finally, cells were acid washed in 100 mM NaCl, 200 mM glycine, pH 2.4 for 20 min at 4°C to remove surface transferrin. Cells were then fixed and processed for IF as described above.

To assay transferrin recycling, cells were labeled to steady state with transferrin conjugated to Alexa 594 as described above. Cells were then washed 3× in DMEM + HB. Cells were next incubated in 5 mg/ml unlabeled human transferrin for 0, 15, 30, or 60 min at 37°C. Finally, cells were acid washed in 100 mM NaCl, 200 mM glycine, pH 2.4 for 20 min at 4°C. Cells were then fixed and processed for IF as described above.

Images were processed to calculate the mean transferrin fluorescence per cell using Image J. Significant differences were assessed using the Student’s t-test. Error bars represent standard error of the mean.

RESULTS

Depletion of β-COP causes fragmentation of the Golgi. We utilized two different siRNA oligos designed to reduce the synthesis of β-COP to assess the role of COP-I in the biogenesis of secretory and endocytic organelles and membrane transport along the secretory and endocytic pathways. HeLa cells were transfected with either a control siRNA or one of the β-COP siRNAs and allowed to proliferate for 24 h, and β-COP levels were assessed by Western blot analysis. Transfection with the β-COP siRNAs resulted in an average 81 or 76% decrease in cellular levels of β-COP, respectively (Fig. 1, B and D). It is likely that complete depletion of β-COP causes cell death and that only partially depleted cells survive for 48 h. Similarly, ldl-F CHO cells held at the nonpermissive temperature for more than 24 h die (34). Therefore, our depleted cells represent a unique hypomorphic system to observe the long-term effects of partial inhibition of COP-I function.

β-COP depletion had minimal effect on levels of other components of the γ/δ/β/ε subcomplex and of the α/ε/β' subcomplex since the levels of the other tested COP-I components remained unaffected (Fig. 1B). Because other subunits of COP-I are not affected by β-COP depletion, we tested whether they remain within a complex. Lysates from control and β-COP-depleted cells were resolved on 5–30% sucrose gradients to separate intact and partial COP-I complexes by size (45, 55). As shown in supplemental Fig. S1A, the COP-I complex migrates similarly in both control and β-COP-depleted cells; supplemental material for this article is available online at the American Journal of Physiology Cell Physiology website. Neither β-COP nor α-COP migrate in lighter fractions that would indicate breakdown of the COP-I into subcomplexes or monomers. Blotting of inputs demonstrates that β-COP was efficiently depleted, whereas α-COP levels are unchanged in β-COP-depleted cells (supplemental Fig. S1A). Therefore, β-COP depletion does not affect the formation of the COP-I complex, and assembled COP-I complexes are present in β-COP-depleted cells.

To explore the cellular function of β-COP, we first compared the Golgi architecture in control and depleted cells by examining the localization of remaining β-COP immunoreactivity. IF microscopy of control and depleted cells taken at the same exposure times shows that all cells retain partial β-COP expression (Fig. 1C). Significantly, even incomplete reduction in β-COP levels leads to dramatic changes in the distribution of β-COP. In control cells, β-COP predominately localizes to the perinuclear Golgi and to ERGIC (Fig. 1C). In contrast, in β-COP-depleted cells, the remaining β-COP relocates to large globular structures distributed throughout the cytoplasm (Fig. 1C). The fragmentation suggests Golgi dispersion, and, in agreement, the globular fragments contain the Golgi marker GM130 (Supplemental Fig. S1B). Quantification of the fragmentation phenotype demonstrates that 92% of cells transfected with the first anti-β-COP siRNA and 77% of cells transfected with the second anti-β-COP siRNA contain fragmented Golgi (Fig. 1E). Since the first anti-β-COP oligo causes higher levels of depletion and Golgi fragmentation, subsequent experiments were performed using that siRNA.

Depletion of β-COP causes the formation of a fragmented compartment containing markers of the ERGIC, Golgi, and TGN. The Golgi is dispersed in β-COP-depleted cells, suggesting a role for COP-I in Golgi biogenesis. However, COP-I has also been shown to be essential for the distribution of ERGIC (41, 63, 70) and has been detected at the TGN (27), although its function there remains unclear. Therefore, we examined the effects of β-COP depletion on the morphology of these compartments. The architecture of the ERGIC was visualized with antibodies against ERGIC-53. ERGIC-53 was redistributed from its normal perinuclear and punctate cytoplasmic localization in control cells (Fig. 2A, ERGIC-53) to large globular structures dispersed throughout the cell (Fig. 2B, ERGIC-53). ERGIC-53 localization in these structures overlapped strongly with the β-COP remaining within the depleted cells. Interestingly, ERGIC-53 distribution in the β-COP-depleted cells
differed from its dispersed localization in BFA-treated cells (compare Fig. 2B and Supplemental Fig. S1C).

As shown in supplemental Fig. S1B, the cis-Golgi is fragmented in β-COP-depleted cells. The morphology of additional Golgi subcompartments was assessed using antibodies against proteins known to reside within different Golgi cisternae. The transmembrane medial-Golgi marker Giantin was redistributed from the characteristic perinuclear Golgi structure in control cells (Fig. 2A, Giantin) to a diffuse ER staining in β-COP-depleted cells (Fig. 2B, Giantin). However, Giantin is also found in β-COP-positive fragments (Fig. 2B, Giantin). The localization is similar to that in BFA-treated cells (compare Fig. 2B and supplemental Fig. S1C, Giantin). Giantin is also observed in both the ER and in globular structures in ldl-F cells depleted of ε-COP by incubation at the nonpermissive temperature (40°C) for 12 h (supplemental Fig. S1D). The localization of another transmembrane medial-Golgi marker Golgin-84 was also altered in β-COP-depleted cells. Golgin-84 relocated from a tight perinuclear Golgi (Fig. 2A, Golgin-84) to the large, peripheral globular structures (Fig. 2B, Golgin-84). This distribution differs from the relocation of Golgin-84 to the ER and small punctate structures in cells treated with BFA (compare Fig. 2B and supplemental Fig. S1C, Golgin-84). Interestingly, it also differs from the localization of Giantin. This suggests that the intracellular trafficking of different proteins may have different requirements for COP-I function.

The architecture of the TGN was also altered in β-COP-depleted cells. The transmembrane TGN protein TGN46 and peripherally associated Golgin-97 both localize to a tight perinuclear TGN in control cells (Fig. 2A). In contrast, TGN46 and Golgin-97 redistribute to β-COP-positive globular structures dispersed throughout the cytoplasm in β-COP-depleted cells (Fig. 2B).

The redistribution of ERGIC, Golgi, and TGN markers into β-COP-positive globular fragments suggests that the compartments may have collapsed into a common structure. Alterna-
tively, each compartment may change in morphology but remain independent and retain its own identity. To differentiate between these possibilities, control and β-COP-depleted cells were costained with combinations of markers for ERGIC, Golgi, and TGN. As shown in Fig. 2C, significant overlap is shown between ERGIC-53 and the Golgi marker Golgin-84. Similarly, GM130, a cis-Golgi marker colocalizes significantly with Giantin-positive fragments. Furthermore, markers for the TGN (TGN46, Golgin-97) show significant overlap with ERGIC (ERGIC-53) and Golgi (Golgin-84) markers (Fig. 2C). Since all markers exhibit significant overlap, it appears that the ERGIC, Golgi, and TGN collapse into a common, fragmented compartment in the absence of COP-I function. Therefore, COP-I is required for both the maintenance of compartment structure and for compartment differentiation.

Anterograde transport of VSV-G and secretory proteins is blocked in β-COP-depleted cells. Since β-COP depletion alters the morphology of the ERGIC, Golgi, and TGN, we assessed the functionality of the secretory pathway by assaying anterograde cargo trafficking. We followed trafficking of a temperature-sensitive mutant of VSV-G (VSV-G ts045) that tracks a cohort of newly synthesized VSV-G from the ER to the plasma membrane. Control and β-COP-depleted cells were transfected with VSV-G and held at the nonpermissive temperature of 39°C to accumulate VSV-G in the ER (3, 60, 77). Cells were then moved to the permissive temperature of 32°C to allow VSV-G egress from the ER and entry into the secretory pathway. Cells were processed to visualize the localization of VSV-G after 0, 2, 6, and 12 h. In control cells, VSV-G is within the ER at 0 h, is concentrated in the Golgi after 2 h, and reaches the plasma membrane after 6 h (Fig. 3A). After 12 h, VSV-G is largely cleared from intracellular stores and is predominantly on the plasma membrane (Fig. 3A). In contrast, in β-COP-depleted cells, the majority of VSV-G remains within the ER after 2 h (Fig. 3B, 2 h). Even after 6 h and 12 h at the permissive temperature, the majority of VSV-G is found in the ER or in large globular β-COP-containing structures (Fig. 3B, 6 and 12 h; two images representing the two most common phenotypes are presented). VSV-G was not detected on the plasma membrane in β-COP-depleted cells, indicating a block in anterograde transport.

COP-I has previously been implicated in secretion of a subset of soluble proteins in yeast (25). Therefore, we assayed protein secretion in control and β-COP-depleted cells. Cells were pulse labeled and chased for various times, and the secretion of newly synthesized proteins was assessed by SDS-PAGE.

Fig. 3. Anterograde transport of vesicular stomatitis virus membrane glycoprotein (VSV-G) is blocked in β-COP-depleted cells. Cells transfected with either control (A) or β-COP siRNA (B) for 32 h were transfected with GFP-VSV-G ts045 and held at the nonpermissive temperature for 16 h to accumulate VSV-G in the ER. Cells were then moved to the permissive temperature of 32°C for 2, 6, and 12 h. At each time point, cells were fixed and processed for IF with anti-green fluorescence protein (GFP) and anti-β-COP antibodies. At 0 h, VSV-G localizes to the ER in both control and depleted cells. In control cells after 6 h, VSV-G is primarily found at the plasma membrane. In β-COP-depleted cells at 2, 6, and 12 h, VSV-G remains either in the ER or in β-COP-positive large globular structures. VSV-G is not detected at the plasma membrane in β-COP-depleted cells.

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PAGE and autoradiography. Cells transfected with control siRNA secrete significant amounts of proteins within 2 h of chase (Fig. 4A, media). A number of proteins are visible with significant secretion of high-molecular-weight species (~200 kDa). In contrast, β-COP-depleted cells appear to secrete only a subset of proteins into the media (Fig. 4A, media). Specifically, there is significant inhibition in the secretion of the high-molecular-weight proteins. The difference in secretion is not due to inhibition of protein synthesis since cell lysates indicate similar levels of radiolabeling in control and β-COP-depleted cells (Fig. 4A, lysate). Western blot analyses of the lysates confirm depletion of β-COP in these experiments (Fig. 4B).

To further characterize the block in trafficking of soluble proteins, we examined the maturation and secretion kinetics of exogenously expressed cochlin (26). Control and β-COP-depleted cells were transfected with a plasmid-containing myc-tagged cochlin. Cells were pulse labeled and chased for various times, and cochlin was immunoprecipitated from media and lysates and analyzed by SDS-PAGE. Cochlin was readily secreted from control but not from β-COP-depleted cells (Fig. 4C, media). Cochlin is a glycoprotein that is synthesized as a 60-kDa precursor and is rapidly processed to the mature 63-kDa form in control cells (Fig. 4C, lysate). In contrast, in β-COP-depleted cells, cochlin is not processed and remains in the lower molecular weight form (Fig. 4C, lysate). The lack of cochlin processing further confirms that depletion of β-COP causes a secretory block.

Markers of the recycling endosomes, but not early endosomes or lysosomes, localize to fragmented, globular compartments in β-COP-depleted cells. Ldl-F cells incubated at the nonpermissive temperature show enlargement of early endosomes and dispersion of lysosomes (12). Therefore, we assessed the effects of β-COP depletion on the morphology of early and late endocytic compartments. In control cells, the early endosome marker early endosome antigen 1 (EEA1) is found in small punctate structures loosely clustered in the perinuclear region (Fig. 5A, EEA1). A similar pattern was observed in β-COP-depleted cells with some of the endosomes appearing slightly enlarged and slightly more dispersed (Fig. 5B, EEA1). Importantly, the EEA1 endosomes clearly remained separate from the β-COP-containing large globular structures. Lysosome-associated membrane protein 1 (LAMP-1), a marker for late endosomes and lysosomes, is clustered in the perinuclear region in control cells (Fig. 5A, LAMP-1). In β-COP-depleted cells, lysosomes are dispersed throughout the cell (Fig. 5B, LAMP-1). Importantly, lysosomes remain separate from the large globular structures containing β-COP. These results indicate that the defects in the architecture of various secretory compartments are not due to a general collapse of all cellular compartments. Rather, they may reflect specific functions that COP-I plays at distinct stages of membrane transport.

Previously, it has been shown that, in ldl-F cells at the nonpermissive temperature, transferrin endocytosis and recycling is decreased (12). Therefore, we analyzed the steady-state localization of the transferrin receptor (TrfR) as a marker for recycling endosomes. In control cells, TrfR localizes to punctate structures distributed throughout the cells and is concentrated in the perinuclear region of the cells (Fig. 5A, TrfR). In β-COP-depleted cells, TrfR no longer distributes in small
Fig. 5. Depletion of β-COP alters the morphology of recycling endosomes and uptake and recycling of transferrin. Cells were transfected with control siRNA (A) or β-COP siRNA (B) for 48 h. Cells were fixed and processed for IF with indicated antibodies. In β-COP-depleted cells, the early endosome marker early endosome antigen 1 (EEA1) appears slightly more dispersed, but does not colocalize with β-COP-containing globular structures. Similarly, a lysosomal marker lysosome-associated membrane protein (LAMP-1) appears more dispersed but does not colocalize with β-COP-containing structures. In contrast, a recycling endosome marker transferrin receptor (TrfR) relocates from the perinuclear region to large globular structures that contain β-COP. Significant overlap exists between markers for recycling endosomes (TrfR) and the TGN (TGN46) in β-COP-depleted cells. C: steady-state uptake of transferrin was assessed in cells transfected with either control or β-COP siRNA for 48 h. Cells were incubated in 50 μg/ml transferrin-Alexa 594 for 1 h, followed by acid washing to remove surface-bound transferrin. Fluorescence images were processed to calculate the mean transferrin fluorescence per cell as described in MATERIALS AND METHODS. β-COP depletion caused a 39% decrease in transferrin endocytosis (P < 0.001). D: transferrin recycling was assessed in cells transfected with either control or β-COP siRNA for 48 h. Cells were labeled to steady-state with transferrin-Alexa 594 for 4 h, followed by acid washing to remove surface-bound transferrin. Fluorescence images were processed to calculate the mean transferrin fluorescence per cell as described in MATERIALS AND METHODS. β-COP depletion caused a slight decrease in the rate of transferrin recycling at 15, 30, and 60 min (P < 0.01). Con, control.

Fluorescence per cell as described in MATERIALS AND METHODS was processed to calculate the mean transferrin fluorescence per cell as described in MATERIALS AND METHODS. β-COP depletion caused a 39% decrease in transferrin endocytosis (P < 0.001). D: transferrin recycling was assessed in cells transfected with either control or β-COP siRNA for 48 h. Cells were labeled to steady-state with transferrin-Alexa 594 for 4 h, followed by acid washing to remove surface-bound transferrin. Fluorescence images were processed to calculate the mean transferrin fluorescence per cell as described in MATERIALS AND METHODS. β-COP depletion caused a slight decrease in the rate of transferrin recycling at 15, 30, and 60 min (P < 0.01). Con, control.

puncta and appears concentrated in large, globular, β-COP-positive structures (Fig. 5B, TrfR). These structures colocalize with the TGN marker, TGN46, indicating that the TrfR is also contained within the common compartment (Fig. 5B).

The unexpected collapse of the recycling endosome into fragmented β-COP-positive compartments led us to assess whether recycling traffic through the collapsed structure was perturbed. Steady-state uptake of transferrin in β-COP-depleted cells was ~60% of that observed in control cells (Fig. 5C). The value is similar to that observed in LDL-F cells at early time points (12). Recycling of transferrin was also slightly but significantly decreased in β-COP-depleted cells at all time points examined (Fig. 5D). Importantly, the remaining transferrin accumulated within the globular compartiments (our unpublished observations), indicating that these compartments are accessible to endocytic traffic, although transport out of the compartment is inhibited.

Cav1 traffic is altered in cells lacking COP-I function. Since newly synthesized VSV-G and endocytosed transferrin both appear to be arrested in the common fragmented compartments in β-COP-depleted cells, we wanted to more directly assay transport of proteins out of this compartment. Cav1 is a small 24-kDa protein that localizes to plasma membrane caveolae, to endosome-like structures termed caveosomes, and to the TGN (see Fig. 6A and Ref. 17). Significantly, it was recently reported that the biosynthetic transport of Cav1 from the Golgi to Cave1 bodies in Caenorhabditis elegans (C. elegans) oocytes occurs in an ARF1-dependent but clathrin-independent manner, consistent with COP-I function (62). The TGN is thought to contain the newly synthesized pool of Cav1 (51, 73, 74). Transport of Cav1 from the TGN to plasma membrane caveolae and caveosomes can be monitored by using cycloheximide to inhibit new protein synthesis since it leads to emptying of the TGN pool of Cav1 (51). The exit of Cav1 from the TGN can be quantified by measuring the level of colocalization between Cav1 and TGN markers before and after adding cycloheximide.

To test role of COP-I in Cav1 traffic, we first compared the steady-state distribution of Cav1 in control and β-COP-depleted cells. In control cells, Cav1 is detected in plasma membrane caveolae, in small punctate structures dispersed throughout the cell, presumably caveosomes, and within the perinuclear region where it colocalizes with Golgin-97, a TGN marker (Fig. 6A). In β-COP-depleted cells, a significant por-
tion of Cav1 is redistributed from cell surface caveolae and caveosomal structures to large, globular compartments containing Golgin-97 (Fig. 6A). This is represented by a 30% increase in the overlap coefficient between Cav1 and Golgin-97 (from 0.51 in control cells to 0.66 in β-COP-depleted cells) (Fig. 6B). The change is not due to changes in Cav1 levels since Cav1 levels are unchanged in control and β-COP-depleted cells (Fig. 6D). Together, the data suggest that Cav1 is trapped in the common compartment in β-COP-depleted cells.

To further explore Cav1 traffic, control and β-COP-depleted cells were treated with cycloheximide for 3 h to prevent new synthesis of Cav1, and the clearance of Cav1 from the globular compartments was assessed by IF. In control cells after cycloheximide treatment, Cav1 immunoreactivity in Golgin-97-positive compartments decreases (Fig. 6A), suggesting a significant proportion of Cav1 traffics out of the TGN within the time frame of this experiment. The overlap coefficient between Cav1 and Golgin-97 decreases from 0.51 to 0.32, representing a statistically significant decrease (Fig. 6B). In contrast, in β-COP-depleted cells after cycloheximide treatment, no significant change in colocalization between Cav1 and Golgin-97 is observed, and Cav1 remains within the globular compartments (Fig. 6, A and B). This suggests that Cav1 does not exit out of the common compartments in β-COP-depleted cells.

Since Cav1 is retained in the common compartment in β-COP-depleted cells, a reduction in Cav1 surface levels is expected. We therefore assayed the steady-state surface levels of Cav1 in control and β-COP-depleted cells. The surface pool of Cav1 can be selectively visualized in methanol-fixed cells stained with the Cav1 P132L mutant (identified in a subset of estrogen receptor-positive breast cancers) (32, 42). Cav1 levels are not affected by β-COP depletion. E: cells were transfected with either control or β-COP siRNA for 48 h. Cells were then lysed and the lysates Western blotted with the indicated antibodies. Cav1 levels are not affected by β-COP depletion. E: cells were transfected with either control or β-COP siRNA for 48 h. Cells were then lysed in the presence of 1% Triton X-100, and the lysates were subjected to equilibrium density centrifugation to isolate lipid rafts (recovered in fractions 4 and 5). Fractions were collected from the gradient and Western blotted with anti-Cav1 antibody. Cav1 is incorporated into lipid rafts in both control and β-COP-depleted cells. IB, immunoblot.

Fig. 6. Biosynthetic transport of caveolin-1 (Cav1) is inhibited in β-COP-depleted cells. A: cells were transfected with either control or β-COP siRNA for 48 h. Cells were then left untreated or treated with 200 μg/ml cycloheximide for 3 h. Cells were fixed and processed for IF with indicated antibodies. In untreated control cells, a significant pool of Cav1 is detected at the TGN and colocalizes with Golgin-97 (Gol97) therein. This pool is reduced after cycloheximide treatment. In untreated β-COP-depleted cells, Cav1 localizes to large globular structures and colocalizes with Golgin-97. This pool of Cav1 remains in the large globular structures after cycloheximide treatment. B: images analogous to those in A were processed (as described in MATERIALS AND METHODS) to obtain the overlap coefficient between Cav1 and Golgin-97. Significant differences were assessed using the Student’s t-test. Error bars represent standard deviation. C: cells were transfected with either control or β-COP siRNA for 48 h. Cells were then fixed using cold methanol and processed for IF to stain surface Cav1. Images were processed to calculate the mean fluorescence per cell using Image J. β-COP depletion caused a slight but significant decrease in Cav1 surface staining (P < 0.02). Significant differences were assessed using the Student’s t-test. Error bars represent means ± SE. D: cells were transfected with either control or β-COP siRNA for 48 h. Cells were then lysed and the lysates Western blotted with the indicated antibodies. Cav1 levels are not affected by β-COP depletion. E: cells were transfected with either control or β-COP siRNA for 48 h. Cells were then lysed in the presence of 1% Triton X-100, and the lysates were subjected to equilibrium density centrifugation to isolate lipid rafts (recovered in fractions 4 and 5). Fractions were collected from the gradient and Western blotted with anti-Cav1 antibody. Cav1 is incorporated into lipid rafts in both control and β-COP-depleted cells.
transport to cell surface caveolae (42). Therefore, the block in exit of Cav1 from the globular compartments in β-COP-depleted cells could be due to altered incorporation into lipid rafts. We used sucrose flotation gradients to examine the incorporation of Cav1 into lipid rafts in control and β-COP-depleted cells (7). In control cells, Cav1 is found in the lipid raft fractions (fractions 4–5) at the top of the gradient, as well as in fractions at the bottom of the gradient containing large membranous elements (Fig. 6E). A similar distribution is observed in β-COP-depleted cells, suggesting that incorporation of Cav1 into lipid rafts is normal in β-COP-depleted cells (Fig. 6E). Our data suggest that Cav1 retention in the large globular structures in β-COP-depleted cells is not due to a lack of incorporation into lipid rafts. Instead, COP-I appears to be required for Cav1 transport.

To confirm the requirement for COP-I function in biosynthetic traffic of Cav1, we utilized ldl-F cells lacking ε-COP. The advantage of the ldl-F cells is that unlike the β-COP depletion, which takes 48 h, the disappearance of ε-COP occurs within 3 h at the nonpermissive temperature. Therefore, effects in ldl-F cells are less likely to be due to indirect effects from prolonged lack of COP-I function. Ldl-F cells or ldl-F cells rescued with a wild-type copy of ε-COP (ldl-F[LDL-F]) were incubated at the permissive temperature (34°C) or the nonpermissive temperature (40°C) for 3 h. This results in the fragmentation of the TGN in ldl-F cells and a less significant fragmentation in the ldl-F[LDL-F] cells (Fig. 7A). In all cells, a significant pool of Cav1 is found at the intact or the fragmented TGN where it colocalizes with TGN marker syntaxin-6 (Fig. 7A). To monitor exit of Cav1, cells at permissive or nonpermissive temperatures were treated with cycloheximide for 3 h at the same temperature (Fig. 7B). The level of colocalization between Cav1 and syntaxin-6 was then determined. In ldl-F cells at the permissive temperature ~58% of Cav1 clears from the TGN within 3 h (Fig. 7C, ldl-F 34°C). In contrast, only ~33% of Cav1 clears from ldl-F cells at the nonpermissive temperature (Fig. 7C, ldl-F 40°C). The exit of Cav1 in ldl-F[LDL-F] cells is rescued at the nonpermissive temperature, with ~58% of Cav1 exiting the TGN in 3 h (Fig. 7C, ldl-F[LDL-F] 40°C). The block in Cav1 clearance in β-COP-depleted cells is significant, confirming that the transport of newly synthesized Cav1 to the plasma membrane is dependent upon COP-I function.

Cav1 interacts with the γ-COP subunit of COP-I. The finding that β-COP is required for Cav1 trafficking suggests that Cav1 may be a COP-I cargo. Therefore, we tested whether Cav1 interacts with the γ-COP subunit of COP-I. γ-COP has previously been shown to mediate binding of COP-I to cargo proteins (4, 31). HeLa cell lysate was immunoprecipitated with antibodies against β-COP and γ-COP. As a negative control, lysate was also immunoprecipitated with antibodies against γ-adapter [a cargo binding subunit of the clathrin coat adaptor AP-1 (6, 35)]. The immunoprecipitated material was then analyzed with Western blot analysis for the coat component and for Cav1, β-COP, γ-COP and γ-adapter subunit of AP-1 were recovered in the respective immunoprecipitates (Fig. 8A). It appears that the solubilization conditions used in our experiments do not retain an intact COP-I complex since γ-COP is not recovered in the β-COP immunoprecipitate and vice versa. Significantly, Cav1 is minimally recovered in the γ-adapter AP-1 immunoprecipitate and is not detected in the β-COP

Fig. 7. Biosynthetic transport of Cav1 is inhibited in ldl-F cells at the nonpermissive temperature. A–B: ldl-F cells and ldl-F[LDL-F] cells rescued with ε-COP were incubated at either the permissive temperature (34°C) or the nonpermissive temperature (40°C) for 3 h. Cells were then left untreated (A) or treated with 200 μg/ml cycloheximide for 3 h (B). Cells were fixed and processed (as described in MATERIALS AND METHODS) to obtain the overlap coefficient between Cav1 and Syn6. In ldl-F cells at the permissive temperature or rescued cells (ldl-F[LDL-F]) at the nonpermissive temperature, this pool is reduced after cycloheximide treatment. In contrast, in ldl-F cells at the nonpermissive temperature, a pool of Cav1 that colocalizes with Syn6 remains after cycloheximide treatment. C: images analogous to those in A and B were processed (as described in MATERIALS AND METHODS) to obtain the overlap coefficient between Cav1 and Syn6. Percent clearance is defined as the percentage of change in the overlap coefficient between cycloheximide-treated and untreated cells. Error bars represent standard deviation.
Immunoprecipitates were analyzed by Western blot analysis with the indicated antibodies against the γ-COP immunoprecipitate (Fig. 8). Cells were lysed under nondenaturing conditions, and the lysates were immunoprecipitated with antibodies against the γ-adaptin subunit of AP-1, β-COP, or γ-COP. Immunoprecipitates were analyzed by Western blot analysis with the indicated antibodies. Cav1 is specifically recovered in the γ-COP immunoprecipitate.

DISCUSSION

Fig. 8. Cav1 interacts with the γ-COP subunit of COP-I. A: control cells were lysed under nondenaturing conditions, and the lysates were immunoprecipitated with antibodies against the γ-adaptin subunit of AP-1, β-COP, or γ-COP. Immunoprecipitates were analyzed by Western blot analysis with the indicated antibodies. Cav1 is specifically recovered in the γ-COP immunoprecipitate.

To confirm our results, we also performed the “reverse” immunoprecipitations in which HeLa cell lysates were immunoprecipitated with antibodies to Cav1 and the immunoprecipitated material was then Western blotted for γ-COP immunoprecipitated with antibodies against LAMP-2 or Cav1. Immunoprecipitates were analyzed by Western blot analysis with the indicated antibodies. Cav1 specifically coimmunoprecipitates with the γ-COP subunit of COP-I.

To confirm our results, we also performed the “reverse” immunoprecipitations in which HeLa cell lysates were immunoprecipitated with antibodies to Cav1 and the immunoprecipitated material was then Western blotted for γ-COP immunoprecipitated with antibodies against LAMP-2 or Cav1. Immunoprecipitates were analyzed by Western blot analysis with the indicated antibodies. Cav1 is specifically coimmunoprecipitated with antibodies against the γ-adaptin subunit of AP-1, β-COP, or γ-COP. As a negative control, lysate was also immunoprecipitated with antibodies against LAMP-2, a known non-COP-I cargo. As shown in Fig. 8B, Cav1 communoprecipitates with γ-COP but not γ-adaptin AP-1. The LAMP-2 communoprecipitates were negative for both coat components and Cav1 (Fig. 8). Together, our results document a specific interaction between Cav1 and γ-COP, suggesting that Cav1 is a COP-I cargo.

In this study, we used siRNA-mediated depletion of β-COP as a means to explore COP-I function. Our partially depleted cells are the first model to show the effects of depletion of a subunit of the β/γ/δ/ε-subcomplex in mammalian cells and provide a unique system in which to assess long-term effects of a lack of COP-I function. Using this model, we have identified two novel processes that require COP-I function: 1) compartmentalization of the ERGIC, Golgi, TGN, and recycling endosomes and 2) transport of Cav1 from the TGN to the cell surface.

Compartment differentiation. We document that depletion of β-COP causes the formation of common, globular compartments containing markers of the ERGIC, Golgi, TGN, and recycling endosomes. This phenotype differs dramatically from organellar changes observed when COP-I function is inactivated by other means. In cells microinjected with anti-β-COP antibodies that stabilize COP-I on membranes, the architecture of the secretory and endosomal compartments is not significantly changed and there is no collapse of compartments into common globular structures (57). Methods that prevent COP-I recruitment to membranes also cause phenotypes that differ dramatically from those in β-COP-depleted cells. In cells treated with BFA (46, 67), expressing dominant negative ARF mutants (13) or expressing dominant negative mutants of ARF guanine nucleotide exchange factor GBF1 (24), COP-I is dispersed within the cytosol and the ERGIC and Golgi collapse into the ER. In those cells, ERGIC and Golgi proteins either redistribute to the ER or relocate to unstable compartments adjacent to ER exit sites.

In β-COP-depleted cells, ERGIC and Golgi proteins are found in common, globular compartments that may be enlarged ERGIC. This is consistent with the peripheral localization of these structures since ERGIC are found dispersed throughout the cytoplasm (66). COP-I has previously been implicated in anterograde transport from ERGIC to the Golgi (1, 47, 53, 63). Transmembrane ERGIC proteins (such as ERGIC-53 and members of the p24 family) and Golgi proteins (such as Giantin and Golgin-84) are known to continuously cycle between the ER and the ERGIC and Golgi compartments (48, 71, 72). If exit from the enlarged ERGIC is inhibited, all of these proteins would be expected to accumulate therein. Inhibition of COP-I function may result in a block in transport from ERGIC to a centrally located Golgi, resulting in enlarged ERGIC-containing markers for the Golgi. The findings suggest that the enlarged ERGIC compartments are arrested and do not support the biogenesis of distal Golgi compartments. Consistent with this idea, an interaction between COP-I and Cdc42 is known to regulate dynemin recruitment to COP-I vesicles, an event required for their transport toward the Golgi (8). Importantly, this interaction occurs through the γ-COP subunit, which is a member of subcomplex 1 of COP-I. Since the remaining subunits of the COP-I complex were shown to remain in a complex, these complexes lacking β-COP may act as dominant negatives that sequester components required to traffic COP-I vesicles along the cytoskeleton.

The presence of common, globular fragments in β-COP-depleted cells is reminiscent of Golgi fragments observed in ldl-F cells grown at the nonpermissive temperature and lacking the ε-COP of the ε/α/β′-subcomplex. Interestingly, both ε-COP and β-COP have been shown to be the subunits within each of the subcomplexes that bind ARF (80, 81). Since binding of COP-I to membranes requires interactions with ARF (52), it is possible that both ε-COP and β-COP subunits regulate membrane association. If these interactions were synergistic, then inactivation of either one would be expected to cause similar cellular phenotypes.

The lack of ERGIC and Golgi differentiation in β-COP-depleted cells suggests that compartmentalization within the secretory pathway occurs in at least two stages with different requirements for COP-I function. The first stage involves the differentiation of a post-ER ERGIC compartment. It is likely that the formation of the ERGIC is initiated by the COP-II coat and requires only a minimal input from the COP-I machinery. In contrast, differentiation of subsequent Golgi compartments is mediated by the COP-I coat and requires more efficient COP-I function. Consistently, COP-II and COP-I have been shown to be recruited and to act in a sequential manner to
regulate transport of protein cargoes from the ER to the Golgi (1, 63, 70). COP-I has also been strongly implicated in retrograde trafficking of proteins (61, 68, 69). Present models propose that COP-I interacts with varying affinities with ERGIC and Golgi proteins and that the avidity of those interactions may define the relative “sorting quotient” for recycling specific proteins to proximal compartments (19, 71). Therefore, the lack of differentiation of the ERGIC and Golgi may be due to both inhibition of COP-I-dependent anterograde transport and retrograde recycling.

Interestingly, depletion of β-COP also appears to cause the collapse of the TGN and recycling endosomes into enlarged ERGIC. The molecular mechanisms that link β-COP depletion to defects in TGN and recycling endosomes may be direct since COP-I has been detected on TGN by immunolocalization (27), and in vitro and in vivo assays have implicated COP-I in endosomal trafficking (12, 78). Furthermore, defects in TGN structure are caused by expression of dominant negative GBF1, the guanine nucleotide exchange factor required for COP-I recruitment (our unpublished results). It is also possible that the localization of TGN46 and TrfR in β-COP-depleted cells may reflect a biosynthetic pool arrested within the enlarged ERGIC. However, this compartment does also appear to maintain characteristics of the original differentiated compartments. Peripheral TGN proteins such as the GRIP-domain protein Golgin-97 and proteins that cycle between the cytoplasm and these compartments, such as the TGN adaptor AP-1 and recycling endosome marker Rab11 (our unpublished observations) are recruited from the cytoplasm to the common compartments.

Importantly, β-COP depletion does not cause collapse of all subcellular compartments. We show that early endosomes, late endosomes, and lysosomes do not collapse into the enlarged ERGIC. This implies a mechanistic distinction between the biogenesis of the Golgi, TGN, and recycling endosomes and the biogenesis of early and late endosomes and lysosomes. Since COP-I function is required for compartmentalization of the ERGIC, Golgi, TGN, and recycling endosomes, our findings support the division of the cellular endomembranes into a subsystem composed of the ERGIC, Golgi, TGN, and recycling endosomes and an independent subsystem composed of early endosomes, late endosomes, and lysosomes.

Traffic progression. Depletion of β-COP also alters the function of the secretory pathway. COP-I has previously been shown to be required for anterograde transport to the Golgi (1, 47, 53, 63). Injection of anti-β-COP antibodies (57, 58) or treatments that prevent COP-I association with membranes (16) inhibit anterograde traffic. We document for the first time that depletion of β-COP also inhibits anterograde traffic. We show that proteins arrest in enlarged globular compartments as shown by the accumulation of exogenously expressed VSV-G or endogenous Cav1.

The effect of β-COP depletion on traffic of secretory proteins is not uniform. It appears that some proteins are secreted while others are retained intracellularly. This is consistent with findings in yeast, where mutations in the γ-COP subunit of COP-I have been shown to inhibit the secretion of only some cargoes (25). The block in protein traffic appears to occur early in the secretory pathway since exogenously expressed cochlin is not terminally glycosylated in β-COP-depleted cells.

We also observed defects in endosomal traffic in β-COP-depleted cells. Specifically, we show a decrease in steady-state transferrin uptake and recycling that is similar to the defects in traffic to and from the recycling endosome in Idl-F cells at the nonpermissive temperature (12). The lower level of endocytosis might be due to reduced numbers of newly synthesized TrfR making it to cell surface. Interestingly, the endocytosed transferrin can enter the globular enlarged common compartment, suggesting that the recycling endosome has fused with the common compartment. The overall defect in transferrin recycling appears to be caused by delayed exit of transferrin out of the common compartment.

It appears that newly synthesized Cav1 destined for the cell surface or caveosomes is also arrested within the common, fragmented compartments. Despite being arrested in these structures, Cav1 incorporation into lipid rafts remains unaffected in β-COP-depleted cells, indicating that the defect in Cav1 transport is not due to a lack of Cav1 oligomerization. We document that Cav1 interacts with γ-COP, a COP-I subunit previously implicated in cargo binding. Our data, together with the observation that biosynthetic transport of Cav1 from the Golgi to Cav1 bodies in C. elegans oocytes is clathrin independent but ARF1 dependent (62), suggest that COP-I regulates biosynthetic transport of Cav1.

The finding that COP-I function is required for biosynthetic transport of Cav1 has strong clinical implications since caveolar transport has been linked to a variety of diseases, including breast cancer, diabetes, obesity, and prion and viral infection (10, 11, 32, 42, 56, 75). Furthermore, Cav1 has been shown to be upregulated in some forms of metastatic prostate cancer and breast cancer, and Cav1 levels correlate with metastasis in esophageal squamous cell carcinoma, clear cell renal cell carcinoma, mammary adenocarcinoma, and lung adenocarcinoma (33, 36, 37, 50, 79). Caveolae-containing lipid rafts provide platforms for signaling of many oncoproteins, including Src and Ras (39). In addition, phosphorylation of Cav1 is thought to play a role in integrin-mediated cell adhesion and signaling (14, 18). Therefore, the identification of COP-I as a regulator of biosynthetic transport of Cav1 and the discovery that the γ-subunit of COP-I interacts with Cav1 may provide a new therapeutic target for the treatment of metastatic cancers.

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