COPII proteins are required for Golgi fusion but not for endoplasmic reticulum budding of the pre-chylomicron transport vesicle

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Summary

The budding of vesicles from endoplasmic reticulum (ER) that contains nascent proteins is regulated by COPII proteins. The mechanisms that regulate lipid-carrying pre-chylomicron transport vesicles (PCTVs) budding from the ER are unknown. To study the dependence of PCTV-ER budding on COPII proteins we examined protein and PCTV budding by using ER prepared from rat small intestinal mucosal cells prelabeled with ^{3}H-oleate or ^{14}C-oleate and ^{3}H-leucine. Budded ^{3}H-oleate-containing PCTVs were separated by sucrose density centrifugation and were revealed by electron microscopy as 142-500 nm vesicles. Our results showed the following: (1) Proteinase K treatment did not degrade the PCTV cargo protein, apolipoprotein B-48, unless Triton X-100 was added. (2) PCTV budding was dependent on cytosol and ATP. (3) The COPII proteins Sar1, Sec24 and Sec13/31 and the membrane proteins syntaxin 5 and rBet1 were associated with PCTVs. (4) Isolated PCTVs were able to fuse with intestinal Golgi. (5) Antibodies to Sar1 completely inhibited protein vesicle budding but increased the generation of PCTV; these changes were reversed by the addition of recombinant Sar1. (6) PCTVs formed in the absence of Sar1 did not contain the COPII proteins Sar1, Sec24 or Sec31 and did not fuse with the Golgi complex. Together, these findings suggest that COPII proteins may not be required for the exit of membrane-bound chylomicrons from the ER but that they or other proteins may be necessary for PCTV fusion with the Golgi.

Key words: Pre-chylomicron transport vesicle, PCTV, COPII proteins, Vesicle budding, Lipid absorption, Sar1, Chylomicrons

Introduction

A major function of intestinal epithelial cells (enterocytes) is absorbing large amounts of dietary lipid. A key step in this process is the movement of nascent triacylglycerol (TAG) from its site of synthesis in the endoplasmic reticulum (ER) to the Golgi complex on its way to exit the cell as a chylomicron. TAG is exported from ER in distinct vesicles, the pre-chylomicron transport vesicles (PCTVs). To examine this process, we developed an in vitro model in which PCTV movement to the Golgi could be measured (Kumar and Mansbach, 1997). PCTVs were isolated and demonstrated to deliver chylomicrons to the Golgi in a vectorial manner (Kumar and Mansbach, 1999). Although this movement required ATP and cytosol, the molecular mechanisms are unknown.

In contrast to PCTV formation, the initial step in ER to Golgi transport of nascent proteins is known and involves the ordered assembly of the COPII proteins Sar1, Sec23/24 and Sec13/31 on the ER membrane (Matsuoka et al., 1998). Sec16, a membrane associated protein, may act as a scaffold for coat assembly. The first step in vesicle formation is the attachment of Sar1, a GTPase, to the ER membrane in its GTP-bound state. The binding of Sar1 to the membrane is phosphorylation dependent (Aridor and Balch, 2000). Sec12, an integral membrane protein, acts as a facilitator of GDP-GTP exchange on Sar1. In its active GTP bound form, Sar1 recruits the Sec23/Sec24 complex. It has been shown that all members of the COPII coat are required for budding and that Sec23/24 mediates the selection of VSV-G, a type 1 membrane protein, as cargo (Aridor et al., 1998). The Sec13/31 protein complex is recruited last and is required for membrane deformation and budding (Aridor and Balch, 2000; Barlowe, 1998; Salama et al., 1997).

Previous studies have demonstrated distinct features of protein and lipid exiting from the ER. Protein transporting vesicles contain COPII proteins on their surface and are 60-80 nm in diameter (Matsuoka et al., 1998). PCTV vesicles are larger (150-500 nm) than the protein vesicles to accommodate the larger size of their cargo, chylomicrons (100-500 nm in diameter (Zilversmit, 1967). To discriminate between vesicle types, they are identified here by their cargo: PCTVs or protein vesicles containing lipid-rich chylomicrons or proteins, respectively. Vesicles containing apoB-48 and nascent TAG generated in the absence of COPII proteins are identified as lipid vesicles.

The present study demonstrates that PCTV generated in

vitro from isolated enterocyte ER meets the criteria for a budded vesicle. The requirements for PCTV budding and the abilities of PCTV to fuse with isolated Golgi were also examined. PCTV generation from isolated ER was dependent on cytosol and ATP. These PCTV were competent to fuse with purified Golgi complexes. To examine the potential role of COPII proteins in PCTV budding, a double-labeling protocol was developed to directly compare budding of nascent protein and PCTV vesicles from enterocyte ER. Antibody depletion of the COPII protein Sar1 or antibody inhibition of Sar1 blocked budding of protein vesicles. By contrast, treatment with either Sar1 or Sec31 antibodies did not inhibit PCTV budding, but enhanced it. Notably, membrane-bound lipid vesicles generated in the absence of COPII proteins were not competent to fuse with isolated Golgi. These observations suggest that the presence COPII or associated proteins are required for PCTV fusion with the Golgi complex, and that membrane-bound lipid particles can bud from the ER in a COPII-independent manner.

Materials and Methods

Materials

Unless otherwise stated, all reagents were procured from Sigma (St Louis, MO). ^{3}H-oleic acid (9.2 Ci/mM), ^{3}H-leucine (180 Ci/mM) and ^{14}C-oleic acid (56 mCi/mM) were obtained from New England Nuclear (Boston, MA). Immunoblot reagents were purchased from Bio-Rad (Hercules, CA). Enhanced chemiluminescence (ECL) reagents were procured from Amersham International (Piscataway, NJ). Protease inhibitor cocktail tablets were obtained from Boehringer Mannheim (Indianapolis, IN). Other biochemicals used were of analytical grade and purchased from local companies.

Antibodies and plasmid

Affinity-purified rabbit polyclonal anti-Sar1 and anti-Sec24 antibodies were provided by J.-P. Paccaud (University of Geneva, Geneva, Switzerland). Rabbit polyclonal antibodies against mammalian Sec31 (Sec31M) have been characterized (Shugrue et al., 1999). Polyclonal antibodies to Sec13 were a gift of Chris Kaiser (MIT, Cambridge, MA). Rabbit polyclonal antibodies to syntaxin 5 were a kind gift from W. E. Balch (Scripps Research Institute, La Jolla, CA). Goat polyclonal anti-apolipoprotein B-48 (apoB-48), apolipoprotein AI (apoA-I), and apolipoprotein A-IV (apoA-IV) antibodies were generously provided by P. Tso, University of Cincinnati (Cincinnati, OH). Mouse monoclonal antibodies to rBet1 and GOS28 were procured from StressGen Biotechnologies (Victoria, Canada). Goat polyclonal anti-calreticulin and -calnexin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit IgG conjugated with agarose beads was purchased from Sigma. Goat anti-rabbit IgG, goat anti-mouse IgG and goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology. His$_6$-Sar1a in a PET 114 plasmid, originating in the laboratory of W. E. Balch, was a generous gift of Brian Storrie (Virginia Polytech Institute, Blacksburg, VA).

Isolation and metabolic labeling of enterocytes

Enterocytes were isolated as described (Kumar and Mansbach, 1997) from the small intestine of male Sprague Dawley rats (200-300 ***g***, Harlan, Indianapolis, IN). ^{3}H-TAG was synthesized from ^{3}H-oleate and sn-2-monooleoylglycerol by the cells as described (Kumar and Mansbach, 1997).

When protein/TAG labeling was required, cells were incubated in buffer A [137 mM NaCl, 1.5 mM EDTA, 11.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.2 mM KCl, 0.5 mM dithiothreitol (DTT) and 10 mM glutamine] with 25 μCi of ^{14}C-oleic acid plus 4 μmol of carrier oleate complexed to BSA, and ^{3}H-leucine (50 μCi) at 37°C for 30 minutes and then placed on ice.

Preparation of ER and cytosol

ER was prepared from radiolabeled enterocytes as described (Kumar and Mansbach, 1997). Rat intestinal cytosol was prepared as described (Kumar and Mansbach, 1997). The cytosol was dialyzed against Buffer B (25 mM Hepes, pH 7.2, 125 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM DTT, and protease inhibitors for 6 hours at 4°C, concentrated fivefold using a 50 ml Amicon Filter with a YM 10 membrane (Amicon, Beverly, MA). This cytosol was further concentrated on a Centricon Filter (Amicon) with a 10 kDa cutoff to 20 mg protein/ml.

In vitro ER budding assay

ER fractions (500 μg protein) in Buffer C (0.25 M sucrose, 30 mM Hepes, pH 7.2, 30 mM KCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 2 mM DTT) were incubated in a total volume of 500 μl containing 50 μl rat small intestinal cytosol (0.8-1 mg protein), and Buffer C with an ATP regenerating system (1 mM ATP, 5 mM phosphocreatine, and 5 U creatine phosphokinase) at 37°C for 30 minutes without Golgi acceptor. The reaction was terminated by placing the reaction tubes on ice. The sucrose concentration of the reaction mixture was adjusted to 0.1 M and the suspension was overlaid on a continuous sucrose gradient and resolved as described (Kumar and Mansbach, 1997).

Depletion of Sar1 from cytosol and ER

To remove Sar1 from cytosol, 20 μl of rabbit polyclonal anti-Sar1 antibody was incubated with 50 μl of intestinal cytosol (1 mg protein), then anti-rabbit IgG conjugated to agarose beads were added. The immunocomplexes were removed by centrifugation. Sar1-depletion was confirmed by immunoblot. To remove Sar1 from the ER, ER was incubated with 2 M urea for 15 minutes at 4°C. The ER was isolated by centrifugation and washed twice in PBS. Sar1 depletion was confirmed by immunoblot.

Removal of Rab proteins

To remove Rab proteins from the ER and cytosol, Rab-GDI was used (Mukherjee et al., 2000). GDP, 1 mM, was pre-incubated with intestinal ER in Buffer C supplemented with the ATP-regenerating system and protease inhibitors for 20 minutes at room temperature followed by the addition of 12.5 μg of Rab GDI (Sigma) for 10 minutes at room temperature (Mukherjee et al., 2000). Removal of Rab proteins from cytosol was accomplished by immunodepletion.

Transport of newly synthesized TAG and apoB-48 to the Golgi

PCTVs (150 μg protein) containing ^{14}C-TAG and ^{3}H-apoB-48 or only ^{3}H-TAG were formed (Kumar and Mansbach, 1997) and incubated with Golgi membranes (300 μg protein), an ATP regenerating system, 500 μg cytosolic protein and Buffer C for 30 minutes at 37°C. The reaction was stopped by adding cold Buffer C and the Golgi fraction obtained by separating the reaction on a sucrose step gradient (Kumar and Mansbach, 1997). ^{3}H-TAG was isolated from the Golgi (Coleman and Bell, 1976).

To demonstrate delivery of PCTV cargo (^{3}H-apoB-48 and ^{14}C-TAG) to the Golgi lumen after fusion, a cis-Golgi enriched fraction was incubated with or without carbonate buffer (Kumar and Mansbach, 1997). The released chylomicron fraction was isolated by centrifugation after adding carrier rat chylomicrons (Mansbach and Arnold, 1986a). The chylomicron fraction was incubated with anti-apoB antibody (Kumar and Mansbach, 1997) and the apoB-48-

antibody complex isolated, washed with PBS and its radioactivity determined. ^{3}H-TAG was extracted separately from the chylomicron fractions (Coleman and Bell, 1976).

To investigate whether PCTV were attached but not fused with the Golgi, ^{3}H-TAG-PCTV (150 μg protein) were incubated with Golgi (300 μg protein) in the fusion assay as above. The ^{3}H-TAG-cis Golgi were isolated using the discontinuous sucrose gradient (Kumar and Mansbach, 1997). The ^{3}H-TAG-cis Golgi was incubated with or without trypsin (0.5 mg/ml) in Hepes pH 7.2 at 4°C for 1 hour (total vol. 1 ml). Soybean trypsin inhibitor and protease inhibitor cocktail were added and the Golgi isolated using the continuous sucrose gradient (Kumar and Mansbach, 1999). The gradient was resolved, 0.5 ml fractions collected, and the total ^{3}H-dpm determined. Fractions 18-21, containing the ^{3}H-dpm, were concentrated using a Centricon filter, and 30 μg protein loaded onto SDS-polyacrylamide gel electrophoresis (SDS-PAGE). GOS28 sensitivity to trypsin was tested by immunoblotting.

Sar1 rescue

Recombinant Sar1 was produced in E. *Coli* transfected with the His_6-Sar1a containing PET114 plasmid. The final product migrated as a single band on SDS-PAGE (silver stain) with an apparent M_r of 27-28 kDa. The band reacted with anti-Sar1 antibody on immunoblot. To test Sar1 rescue of Sar1-depleted cytosol and ER, urea washed ER (500 μg prot, containing ^{14}C-TAG and ^{3}H-protein) was incubated with cytosol (1 mg protein), recombinant Sar1 (40 μg prot), an ATP regenerating system, and E600 (0.025%) in 500 μl total volume for 30 minutes at 37°C. The reaction was placed on a sucrose density gradient as before and the gradient resolved. ^{14}C-TAG-dpm and TCA precipitated ^{3}H-protein-dpm were determined in each tube.

Measurement of TAG and protein radioactivity

TAG radioactivity was quantitated as described (Kumar and Mansbach, 1997). Protein radioactivity was measured after precipitation with trichloroacetic acid (TCA). In experiments in which both ^{3}H- and ^{14}C-radiolabels were used, the scintillation analyzer (Packard TriCarb, Packard Instrument, Downer's Grove, IL) was set in the dual-isotope mode.

Gel electrophoresis and immunoblots

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted onto nitrocellulose membranes (Bio-Rad) as described (Kumar and Mansbach, 1997). Proteins were detected by developing the blots using ECL and exposing the developed blots to Biomax film (Eastman Kodak, Rochester, NY).

Electron microscopy

To visualize budded vesicles by negative staining, glow-discharged Ni-grids were coated with carbon and formvar (Mukherjee et al., 2000). The coated side of the grid was placed on top of a drop of PCTV, ≈1 mg protein, and incubated for 2-3 minutes and rinsed with PBS and H_2O. Samples were stained with 0.5% aqueous uranyl acetate for 1 minute, blotted on filter paper, air dried and examined under a JEOL 1200 EX electron microscope at 12,000× (JEOL, Peabody, MA).

To examine PCTVs at thin section electron microscopy (EM), PCTV were collected from 3 to 4 experiments and concentrated using a Centricon filter with a YM 10 membrane. The buds, ≈1 mg protein, were pelleted (2500 ***g*** for 10 minutes in an Eppendorf table top centrifuge at 4°C), fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide buffered with 0.2 M imidazole, pH 7.2, and embedded in Spur medium. Sections were cut using a glass knife, stained with uranyl acetate and lead citrate (Tipton et al., 1989) and examined using a JEOL 1200 EX electron microscope at 5000× magnification.

Immunolabeling of the PCTVs was performed as described (Mukherjee et al., 2000). Briefly, samples were incubated with 10% BSA containing mouse anti- (1:100) and rabbit anti-syntaxin-5 (1:100) antibodies for 3-4 hours and subsequently with goat anti-mouse IgG (1:50) conjugated with 10 nm colloidal gold and goat anti-rabbit IgG (1:50) conjugated with 15 nm colloidal gold. The samples were fixed in 1% glutaraldehyde in PBS for 10 minutes and stained with 0.5% aqueous uranyl acetate for 1 minute, and examined under the JEOL 1200 EX electron microscope (12,000×). Control studies were performed using anti-mouse and rabbit pre-immune IgG and processed as described.

Measurement of lipid-P

Lipid phosphorus (lipid-P) was measured by extracting the phospholipids from cellular fractions (Folch et al., 1957), and measuring the lipid-P content by chemical means after digestion of the phospholipids by H_2SO_4 as described (Knox et al., 1991).

Statistical analysis

Comparisons between means were carried out using a statistical package supplied by GraphPad Software (Instat, GraphPad Software, San Diego, CA) using a two-tailed *t*-test.

Results

Establishment of the PCTV-budding assay

To examine the formation of PCTVs from ER, enterocyte ER was radiolabeled with ^{3}H-oleate to form ^{3}H-TAG. To initiate budding, radiolabeled ER was incubated at 37°C for 30 minutes in the presence of cytosol and ATP. This did not change the appearance of the ER at EM (data not shown). The reaction mix was separated on a continuous sucrose density gradient (0.1 M-1.15 M sucrose) and centrifuged (82,000 ***g*** for 90 minutes). Prior work from our laboratory has shown that PCTVs have a low buoyant density (Kumar and Mansbach, 1999) and float toward the top of the gradient. The heavy region of the gradient contained both the donor ER and most of its associated lipid label and was excluded from analysis. However, we estimate that 12-15% of the nascent TAG shifted from the ER to the PCTV fraction during a 30 minute incubation compared with 12-14% of newly synthesized protein consistent with prior results (Rexach et al., 1994).

To observe vesicle formation, we examined concentrated putative PCTV fractions (fractions 1-7, Fig. 2A) by EM using negative staining. The results (Fig. 1A), show a field of pure vesicles ranging in size from 142 to 350 nm.

Transmission electron microscopy of thin sections demonstrated that PCTVs appear as 350-500 nm structures containing osmiophilic chylomicrons (Fig. 1B). Some of these structures appear elongated, which may be an artifact of the preparation process, may be due to vesicle fusion or may indicate that these structures are part of the vesicular tubular complex (VTC) (Saraste and Svensson, 1991). The latter postulate is supported by the presence of the VTC marker p58 (rodent homologue of ERGIC-53) (Saraste and Svensson, 1991) in this fraction (see below, Fig. 5). Putative protein vesicles (Fig. 1B, inset) were found in the mid-portion of the gradient (collected and concentrated from fractions 9-12, Fig.

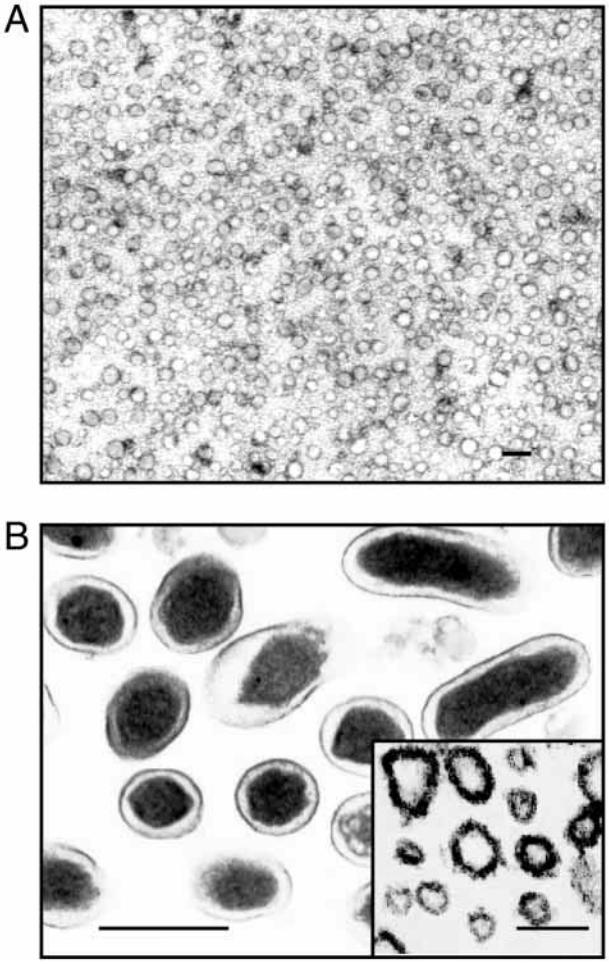

Fig. 1. (A) Negative stained EM of PCTVs generated from intestinal ER. PCTVs (fractions 1-7 from Fig. 2A) were formed from intestinal ER and examined by EM. All the vesicles appear intact and range in size from 142-347 nm (12,000×). The bar represents 500 nm. (B) Thin section transmission microscopy of PCTVs and protein vesicles (inset). PCTVs and protein vesicles were stained and processed for thin section EM (5000×). PCTVs are 350-500 nm in size and contain osmiophilic lipid. Bar, 500 nm. (Inset) Protein vesicles (fractions 9-12 from Fig. 2C, prepared for EM appeared as ≈70-117 nm in diameter and lacked lipid. Bar, 100 nm. For details, see Materials and Methods.

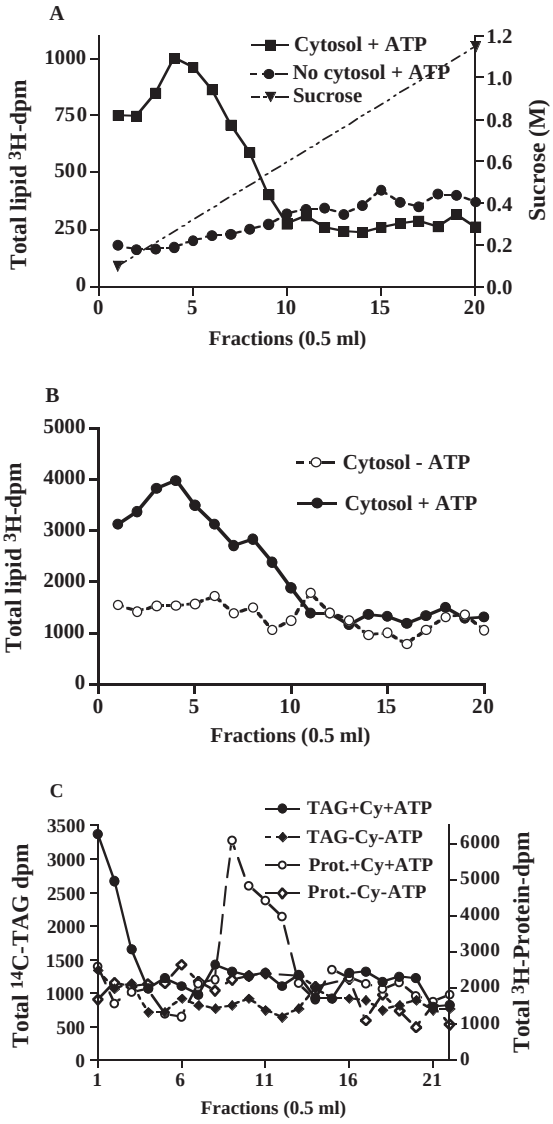

Fig. 2. Effect of cytosol and ATP on PCTV and protein vesicle budding from ER. (A) ER containing ^{3}H-TAG was incubated in the presence (+) or absence (–) of intestinal cytosol. A continuous sucrose gradient (0.1-1.15 M) was used to isolate the PCTVs. The total ^{3}H-dpm from each fraction (150 μl of 500 μl total volume) was counted. (B) A similar experiment to A was performed with (+) or without (–) ATP and an ATP generating system. The gradient was resolved as in A and total ^{3}H-dpm determined. In A and B only fractions 1-20 of 70 total fractions (total tube volume, 35 ml) are shown since no ^{3}H-dpm above baseline were found in fractions 21-70. (C) ER containing ^{14}C-TAG and ^{3}H-protein were incubated with and without cytosol in the presence of an ATP-generating system. The reaction mix was resolved on the sucrose gradient using 11 ml centrifuge tubes and fractions of 0.5 ml collected.

2C). They had an irregular coat, contained little electron dense material and were smaller (70-117 nm diameter) than PCTVs.

Fig. 2A shows the distribution of ^{3}H-dpm (85% of the dpm are TAG, the remainder are partial glycerides and FFA) across the sucrose gradient. ^{3}H-dpm was maximal in the initial fractions, which suggests the presence of PCTVs. The generation of the light fraction required the addition of both ATP and cytosol to the ER (Fig. 2B). However, the generation of the low density fraction did not require added calcium (^{3}H-lipid in PCTV fractions: 5 mM Ca=9259 ^{3}H-dpm; 1.8 mM Ca=11,714 ^{3}H-dpm; 0 mM Ca=13,603 ^{3}H-dpm; values are means of two experiments). The appearance of ^{3}H-dpm was blocked by incubation at 4°C (not shown). To simultaneously examine protein and PCTV budding from intestinal ER preparations, we generated enterocyte ER with ^{3}H-proteins and ^{14}C-TAG. Fig. 2C shows newly synthesized proteins, as indicated by their ^{3}H-dpm radioactivity, in the middle of the gradient (fractions 9-12). Little newly synthesized protein was found in fractions 1-3 occupied by PCTV. Most ^{3}H-proteins were present in the densest portion of the gradient with the ER (the ER pellet data are not shown). By contrast, ^{14}C-dpm associated with TAG was localized to the light fraction of the

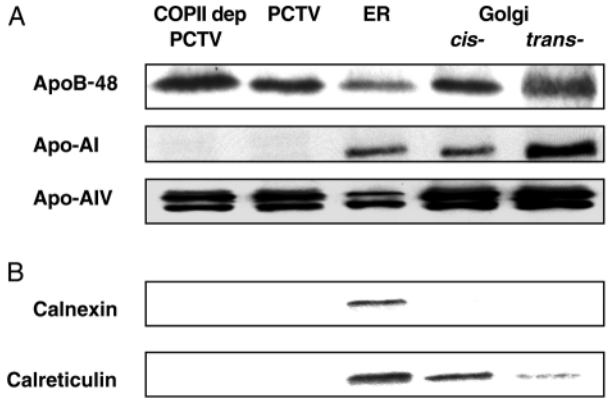

Fig. 3. Immunoblots of subcellular fractions using antibodies to apoB-48, apoA-I, apoA-IV (A), and calnexin and calreticulin (B). 30 μg of protein from each fraction were separated on 7.5% (for apoB) or 10% (for other indicated proteins) SDS-PAGE.

gradient as expected for PCTV with few ^{14}C-dpm in the mid-portion of the gradient associated with the protein vesicles.

A characteristic of chylomicrons, which are contained in PCTVs, is the presence of apoB-48 and apoA-IV (Kumar and Mansbach, 1997). The immunoblot of PCTVs demonstrates the presence of both apolipoproteins (Fig. 3A). ApoA-I is associated with mature chylomicrons but is not present in PCTVs, which confirms previous studies (Kumar and Mansbach, 1999). Calnexin and calreticulin, two ER-resident proteins (Bergeron et al., 1994; Smith and Koch, 1989), were not detected in the PCTV fraction (Fig. 3B). Small amounts of calreticulin, but not calnexin, are also present in the Golgi fraction. These data are consistent with the known presence of calreticulin in the Golgi (Zuber et al., 2000) or could be due to the crossreactivity of the carboxyl end of calreticulin (to which the antibody was directed) with CALNUC, a Golgi-resident protein localized primarily in the cis Golgi (Pin et al., 1998). The lack of calreticulin in the PCTVs suggests that ER luminal resident proteins are not included as cargo in PCTVs.

To examine the potential of PCTVs to fuse with the Golgi, PCTVs loaded with ^{14}C-TAG and ^{3}H-apoB-48 were incubated with unlabeled acceptor Golgi. In the absence of cytosol, there was little TAG or apoB-48 dpm associated with the Golgi (Fig. 4A). Similarly, when PCTVs were incubated with Golgi at 4°C, no increase in ^{3}H-TAG dpm was found in the Golgi in excess of the ^{3}H-TAG dpm present in the absence of cytosol (1618 vs. 1779 ^{3}H-dpm, mean of two experiments). When PCTV was incubated with Golgi in the presence of cytosol, ^{14}C and ^{3}H increased four- to fivefold in a Golgi fraction (Fig. 4A). Most of the ^{14}C-TAG and ^{3}H-apoB-48 were associated with the Golgi since the majority of the dpm remained with the Golgi membranes in the absence of carbonate (Fig. 4B). Carbonate treatment, which disrupts the Golgi membranes, released most of the ^{14}C-TAG and ^{3}H-apoB-48 to the top of the gradient where carrier chylomicrons were found (Fig. 4B). When the fusion reaction was performed at 4°C instead of 37°C, no shift of the TAG to the Golgi fraction was observed (with cytosol, 3936 ^{3}H-TAG-dpm; without cytosol, 1779 ^{3}H-TAG-dpm; and with cytosol incubated at 4°C, 1618 ^{3}H-TAG-dpm were transferred from PCTVs to the cis-Golgi during the 30 minute incubation; means of two experiments).

To release any PCTVs that might be attached by protein linkers but not fused with the Golgi, PCTVs were incubated with Golgi, and then the Golgi was re-isolated and treated with trypsin. After protease treatment, protease inhibitors were added and the Golgi and other fractions were isolated on a continuous sucrose gradient. The lack of protease-dependent PCTV release to the light part of the gradient after protease treatment suggests that they are not tethered to the Golgi complex (Fig. 4C). Further, the location of the ^{3}H-dpm was consistent with the distribution of the Golgi as shown by the specific Golgi SNARE, GOS28 (Subramanian et al., 1996), in this portion of the gradient (Fig. 4C, inset). The reduced GOS28 signal on immunoblot confirmed the efficacy of the trypsin treatment (Fig. 4C, inset). These data indicate that PCTV are fusion competent and deliver pre-chylomicrons to the Golgi lumen.

To confirm that PCTVs were formed by budding from the ER and not its fragmentation, several studies were performed. First, ER, PCTVs, protein vesicles and Golgi proteins were immunoblotted with antibodies to syntaxin 5, a membrane protein important in vesicle targeting to the Golgi (Dascher et al., 1994), and rBet1, a v-SNARE (Zhang et al., 1997). Signals were present in ER and Golgi fractions, but they were much less prominent than those observed in PCTVs and protein vesicles (Fig. 5A). The immunoblots shown in Fig. 5A were loaded with the same amount of protein in each lane. To assess the membrane content of each fraction, lipid-P was assayed. The results were: PCTV=0.16; ER=9.5; cis-Golgi=20.7; and trans-Golgi=53.3 μg lipid-P/mg protein (means of two experiments). Thus the differences shown in the blots between ER, Golgi and PCTV would be magnified if they were based on lipid-P rather than on protein. Second, the distribution of key markers was examined in all sucrose density gradient fractions collected after the addition of ER, cytosol and ATP and compared with the originating ER and cytosol. As shown in Fig. 6, Sar1, rBet1 and Sec31 all produced peaks that were coincident with both PCTVs and protein vesicles (Fig. 2C). As expected, apoB-48 was found only in the light fraction of the gradient in the location of PCTVs. Calnexin was not detected in either vesicle fraction but was present in the original ER and cytosol (TM). These data strongly suggest that Sar1, Sec31 and rBet1 were recruited to PCTVs as well as to protein vesicles and were not products of ER fragmentation.

Next, we examined the protease sensitivity of the PCTV cargo protein, apoB-48. When PCTVs were treated with proteinase K there was no reduction in apoB-48 signal on immunoblot, compared with PCTVs not exposed to the proteinase (Fig. 5B). By contrast, when PCTVs were incubated with proteinase K and Triton X-100 to dissolve the vesicle membrane, the apoB-48 signal was eliminated. These studies provide biochemical evidence that PCTVs have an intact membrane that is acquired during budding.

To detect specific cargo proteins, fractions were probed for p58 immunoreactivity (Fig. 5A) (Rowe et al., 1996). A moderate signal was detected in the ER, Golgi and PCTV, and the most intense reaction was in the area of the sucrose gradient expected for protein vesicles. One interpretation of these findings is that both PCTVs and protein vesicles contain p58.

Role of COPII proteins in PCTV-budding from ER

The budding of nascent protein vesicles from the ER requires

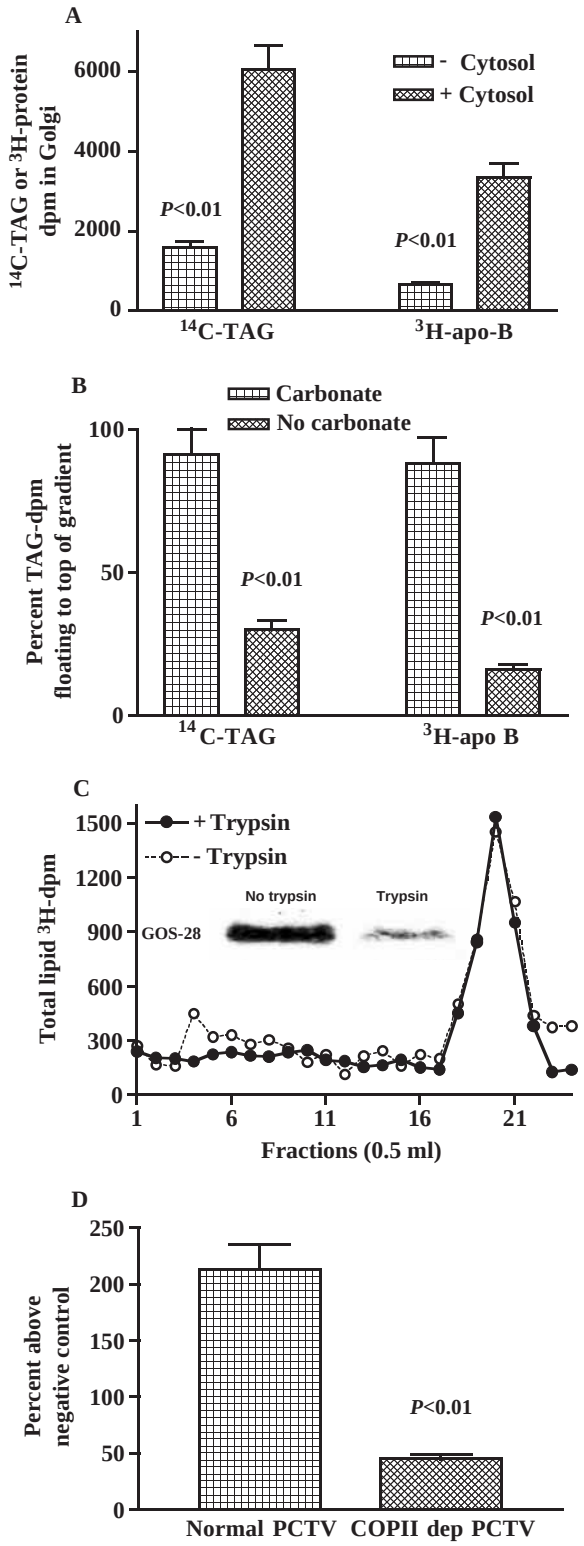

Fig. 4. Transport to the Golgi and delivery to the Golgi lumen of newly synthesized ^{14}C-TAG and ^{3}H-apoB-48 in PCTVs. (A) PCTVs (150 μg prot) containing ^{14}C-TAG and ^{3}H-apoB-48 were incubated with non-radiolabeled Golgi (300 μg prot) in the presence (+) or absence (–) of cytosol (0.5 mg prot). The Golgi was separated from PCTVs and the Golgi ^{14}C-TAG extracted. The Golgi ^{3}H-apoB-48 was isolated by immunoprecipitation. Of the total ^{14}C-TAG-dpm and ^{3}H-apoB-48-dpm, 28% and 25%, respectively, were delivered to the Golgi during the incubation. (B) After the fusion reaction, the Golgi was separated from PCTV on a sucrose gradient and incubated with or without carbonate (100 mM, pH 11) as indicated to release the chylomicrons. Chylomicron ^{14}C-TAG was extracted, and its ^{3}H-apoB-48 isolated by immunoprecipitation. The data are the means of three experiments. (C) ^{3}H-dpm-PCTVs (150 μg prot, 30,640 dpm) were incubated with Golgi membranes (300 μg prot). The Golgi were isolated and treated with (+) or without (–) trypsin (0.5 mg/ml final concentration) for 1 hour at 4°C. Trypsin inhibitors were added and the Golgi isolated. ^{3}H-dpm was determined for each fraction. (Inset) 30 μg protein from fractions 18-21 from the incubations with or without trypsin were loaded onto SDS-PAGE and immunoblotted for GOS28. (D) The fusion of ^{3}H-TAG-PCTV generated in native cytosol (Normal PCTVs) or Sar1-depleted cytosol (COPII dep PCTV) with Golgi. The Golgi was isolated and ^{3}H-TAG extracted. The data are the mean±1 s.e.m. (n=3). For details, see Materials and Methods.

only the COPII proteins Sar1, Sec23/24 and Sec13/31 (Barlowe, 1998). As shown by the immunoblots in Fig. 5C (see Fig. 9 for Sec24), each COPII protein was present in PCTVs. Sar1, Sec 13 and Sec31 appeared greater than tenfold more concentrated in PCTVs compared with the ER. In the case of Sar1, Sec13 and Sec31, the PCTVs were loaded at one-third the amount of protein as the other fractions. The presence of the COPII proteins on the PCTV vesicles suggested that COPII proteins might mediate their budding.

To test the functionality of COPII proteins in PCTV budding, cytosol (0.8 mg protein) was pre-incubated with anti-Sec31 antibodies (5 μl) at 4°C for 1 hour and the treated cytosol used in our budding assay. The results are shown in Fig. 7. Instead of the expected inhibition of budding, an increase in budding of approximately sixfold occurred. Addition of pre-immune rabbit IgG had no effect (Fig. 7).

To remove any residual Sec31 from the ER membrane that might contribute to budding activity, the ER was washed with 2 M urea (data not shown). Although urea-treated ER was not as effective as native ER for budding, anti-Sec31-antibody-treated cytosol continued to induce greater amounts of vesicles than cytosol treated with pre-immune IgG (no cytosol, 5805±612 ^{3}H-dpm; with cytosol + pre-immune IgG, 15,110±1456 ^{3}H-dpm; with cytosol + anti-Sec31 antibody, 70,695±5927 ^{3}H-dpm; data from pooled fractions 1-7 of the sucrose gradient±s.e.m., n=4). These results show that lipid vesicle budding can occur in the absence of Sec31 activity, a protein that is required for COPII-dependent budding, but does not establish the functionality of these vesicles.

The effects of the COPII protein, Sar1 (Barlowe et al., 1993; Matsuoka et al., 1998), on PCTV budding were next examined. Immunoblots of PCTVs, ER and intestinal cytosol showed the presence of Sar1 in the cytosol, a smaller amount in PCTVs, and very little in the ER (Fig. 5C). To examine Sar1 function, the protein was depleted from cytosol (Fig. 9A) prior to the budding assay. Similar to Sec31-antibody-treated cytosol, Sar1-depleted cytosol (closed squares) markedly enhanced lipid vesicle budding (Fig. 8A). Mock-depleted cytosol was used as the source of native cytosolic protein (open squares, Fig. 8A). When Sar1-depleted cytosol was used, no Sar1 was detectable on lipid vesicles by immunoblot (Fig. 9B), whereas it was easily identified in native cytosol as well as native ER (Fig. 9A). Because Sar1 was present in native ER, the ER was washed with

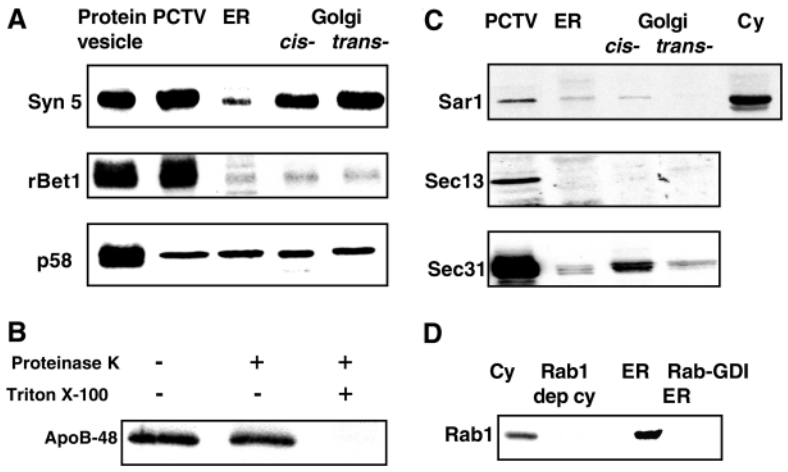

Fig. 5. (A) PCTVs and protein vesicles concentrate SNARE proteins as analyzed by immunoblotting of different subcellular fractions for syntaxin 5 and p58. Proteins (30 μg from each fraction) were separated on 12% SDS-PAGE and immunoblotted as described. (B) The effect of protease treatment on a PCTV cargo protein. PCTVs were incubated with proteinase K (0.5 mg/ml) in the presence or absence of 1% Triton X-100 for 30 minutes at 4°C. 30 μg of protein from treated or untreated PCTVs were used for immunoblotting for apoB-48. (C) Immunoblots of subcellular fractions for Sar1, Sec13 and Sec31. Proteins (30 μg for each fraction except PCTVs, 10 μg) from the different subcellular fractions were separated on 12% SDS-PAGE (Sar1 and Sec13) and 10% SDS-PAGE (Sec31), followed by immunoblotting as in Fig. 2. (D) Immunoblot showing depletion of Rab1 from cytosol (Rab1 dep cy) by using anti-Rab1 antibodies and depletion of Rab1 from the ER (Rab-GDI ER) by treatment with Rab-GDI. For details, see Materials and Methods.

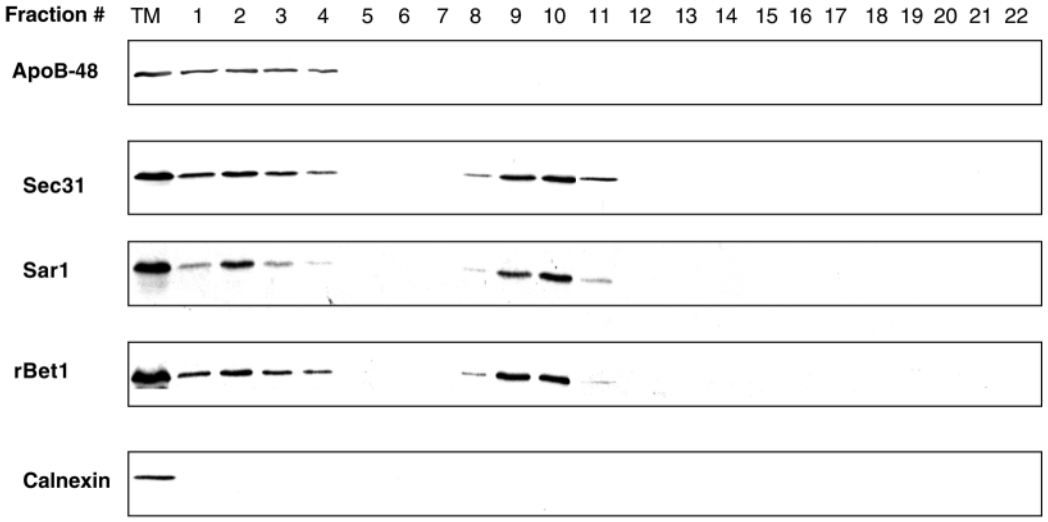

Fig. 6. Immunoblot showing the distribution of Sar1, Sec31, rBet1, apoB-48 and calnexin across a sucrose gradient. ER, cytosol and an ATP regenerating system were incubated for 30 minutes at 37°C and placed on a sucrose gradient as in Fig. 2C. The gradient was resolved and an equal volume (100 μl) of each fraction and 20 μl (60 μg protein) of the original ER and cytosol mixture (TM) were separated on SDS-PAGE. The gel was transblotted and probed with antibodies to the indicated proteins.

2 M urea to remove the Sar1 (Fig. 9A), and tested for its ability to generate lipid vesicles using cytosol treated with Sar1 antibodies (Fig. 8B). An increase in budding of approximately tenfold was observed over cytosol to which pre-immune IgG had been added (Fig. 8B). Notably, lipid vesicles formed in Sar1-depleted cytosol lacked not only Sar1, but also Sec24, Sec31 and p58 (Fig. 9B). However, they were similar to PCTVs formed from non-depleted cytosol in that they contained apoB-48 and apoA-IV (Fig. 3A), they appear to concentrate both apolipoproteins by comparison with the ER (Fig. 3A), and lipid vesicle apoB-48 was resistant to proteinase K treatment unless Triton X-100 was added (Fig. 9C). That proteins were removed from PCTVs by the antibody treatment was suggested by the increase of lipid-P per milligram of protein in Sar1-depleted lipid vesicles (0.47 μg lipid-P/mg protein) compared with PCTVs (0.16 μg lipid-P/mg protein, means of two experiments). Importantly, syntaxin 5 and rBet1 were present in lipid-rich vesicles formed in native or Sar1-depleted cytosol (Fig. 9B). Finally, binding of Sar1 to ER membranes was inhibited by H89 as described (Aridor and Balch, 2000) (Fig. 9B). Proteins other than Sar1 were not sought in PCTVs using H89-treated cytosol since we wished only to be certain that H89 blocked Sar1 binding to PCTVs. Although H89 removed Sar1 from PCTVs (Fig. 9B), the treatment did not affect PCTV budding (Table 1).

Table 1. The effect of H89, GTPγS and Rab depletion on PCTV formation from ER

Addition	Lipid ^{3}H-dpm*
No cytosol	2857±261
Plus cytosol†	15,056±1602
H89-treated cytosol (2 μM)†	15,050±1583
Rab-GDI‡	15,091±1458

*The data are the mean±s.e.m. (n=4).

†1 mg of cytosol protein was incubated with 500 μg ER protein prelabeled with ^{3}H-TAG in the presence of an ATP regenerating system. PCTVs were allowed to form and were separated on a continuous sucrose density gradient. The data (mean±s.e.m., n=4) are the sum of the ^{3}H-dpm from the first seven fractions.

‡Samples were processed as described above but the cytosol was immunodepleted of rab1 and the ER was pre-treated with 12.5 μg of Rab-GDI to remove rab1 (for details, see Materials and Methods).

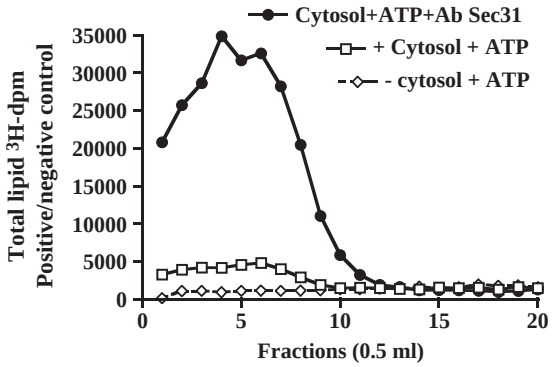

Fig. 7. The effect of Sec31 antibodies on PCTV budding. The budding assay was performed in the absence (–) or presence (+) of native cytosol (1 mg protein) plus pre-immune IgG and cytosol (1 mg protein) treated with 3 μl of anti-Sec31 antibodies (Sec31). Data from fractions 1-20 are shown since fractions 21 to 70 had no ^{3}H-dpm over baseline.

Together, these findings suggest that lipid vesicles that share characteristics with PCTVs can form in the absence of COPII proteins. However, a major difference between PCTVs formed in native cytosol and the lipid vesicles formed under Sar1-depleted conditions is that the latter are no longer fusion competent with the Golgi (Fig. 4D). One possible explanation for this lack of fusion is the absence of p58 on lipid vesicles generated in the absence of COPII proteins. The p58 protein is required for recruitment of COPI proteins that mediate fusion with the Golgi and, as shown here (Fig. 9B), p58 is not present on lipid vesicles formed in Sar1-depleted cytosol (Tisdale et al., 1997).

The lipid vesicles generated in the absence of Sar1 were examined at EM by negative staining. As shown in Fig. 10A, they were morphologically identical to the PCTVs seen in Fig. 1A. Thin section EM of the lipid vesicles (Fig. 10B) also demonstrated morphology similar to that shown in Fig. 1B in which budding occurred with native cytosol. Finally, to demonstrate that the lipid vesicles formed in the absence of

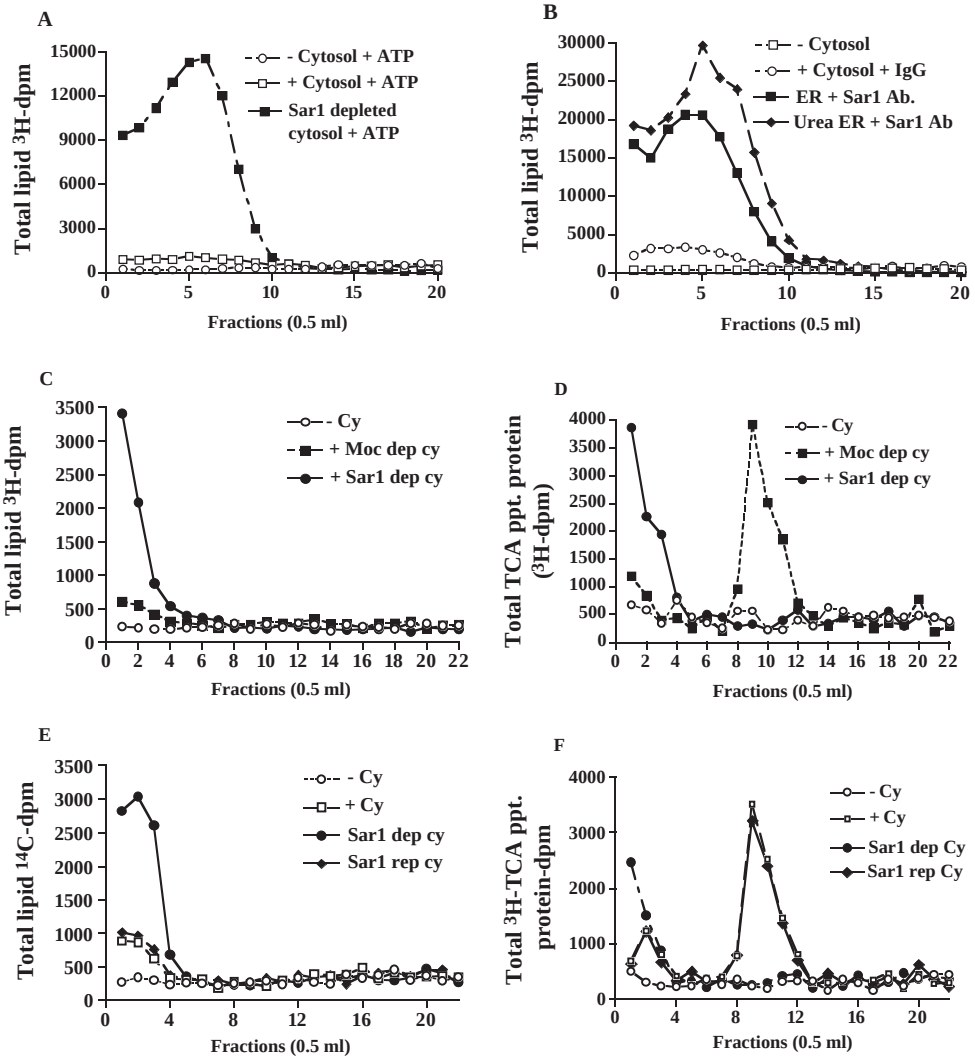

Fig. 8. The effect of Sar1 antibodies and Sar1-depleted cytosol on PCTV (A,B,C, E) and protein vesicle (D,F) budding from ER. (A) The effect of Sar1-depleted cytosol (0.8 mg protein) on PCTV-budding. Native ER was incubated with (+) or without (–) cytosol and compared with ER incubated with Sar1-depleted cytosol (0.8 mg protein). Total ^{3}H-dpm was determined for each fraction. (B) Cytosol (1 mg protein) was (+) or was not (–) included in the budding assay. Pre-immune IgG was added to the native cytosol (+ Cytosol + IgG). Native ER was incubated with cytosol to which Sar1 antibodies had been added (ER + Sar1 Ab.) Native ER was treated with 2 M urea and incubated with cytosol to which Sar1 antibody had been added (Urea ER + Sar1 Ab). The total ^{3}H-dpm is shown on the ordinate. For A and B, 35 ml centrifuge tubes were used. Data from fractions 1-20 are shown since fractions 21 to 70 had no ^{3}H-dpm over baseline. (C,D) The effect of Sar1-depleted cytosol on PCTV and protein vesicle budding. ER containing ^{14}C-TAG and ^{3}H-protein was immunodepleted of Sar1 (+ Sar1 dep cy), mock depleted cytosol (Moc dep cy) or no cytosol (– Cy). The reaction mix was resolved on a sucrose gradient using 11 ml centrifuge tubes and 0.5 ml fractions obtained. The ^{14}C-dpm-TAG (C) and TCA precipitable ^{3}H-dpm-protein (D) were determined for each fraction. (E,F) The effect of recombinant Sar1 protein on PCTV and protein vesicle budding in the presence of Sar1-depleted cytosol. Experiments similar to C and D were performed using ^{14}C-TAG and ^{3}H-protein-labeled ER incubated with no cytosol (– Cy), mock depleted cytosol (+ Cy), Sar1-depleted cytosol (Sar1 dep cy) or Sar1-depleted cytosol supplemented with recombinant Sar1 protein (Sar1 rep cy). ^{14}C-TAG-dpm (E) and ^{3}H-protein-dpm (F) were determined.

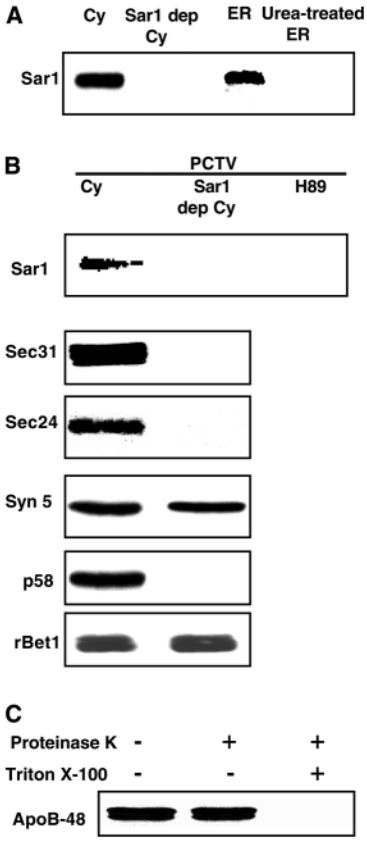

Fig. 9. Effect of Sar1-depleted cytosol on the presence of COPII proteins in PCTVs. (A) Native cytosol (Cy), Sar1 immunodepleted cytosol (Sar1 dep Cy), native ER, and ER washed with 2 M urea (Urea-treated ER) (30 μg protein each) were probed with anti-Sar1 antibodies. (B) PCTVs (30 μg protein), formed under different conditions as indicated, were used for immunoblots as in Fig. 2. PCTVs formed in native cytosol (Cy) or from Sar1 immunodepleted cytosol (Sar1 dep Cy) or in the presence of H89 (H89) were probed with anti-Sar1 antibodies. PCTVs (30 μg protein) formed in native cytosol (Cy) or Sar1 immunodepleted (Sar1 dep Cy) cytosol were probed with antibodies to Sar1, Sec24, Sec31, syntaxin 5, p58 and rBet1. (C) The effect of protease treatment on PCTV cargo (apoB-48) generated in Sar1-depleted cytosol. PCTVs generated in Sar1 depleted cytosol were incubated with proteinase K (0.5 mg/ml) as in 5B. 30 μg protein of protease treated or untreated PCTVs were separated on 7.5% SDS-PAGE and immunoblotted with rabbit polyclonal anti-apoB-48 antibodies. For details, see Materials and Methods.

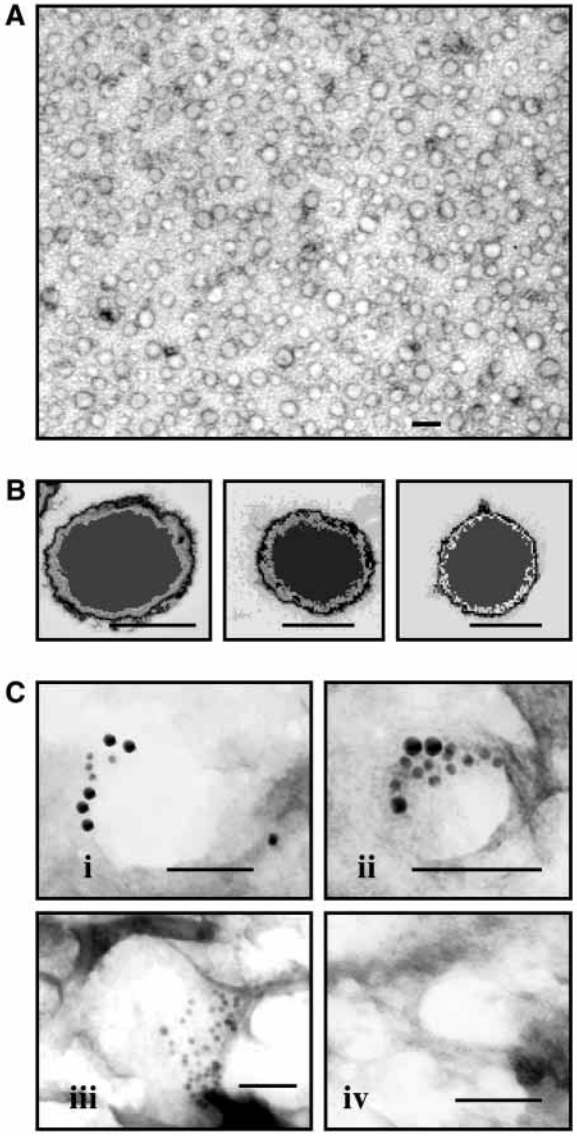

Fig. 10 Negatively stained and thin section electron microscopy of PCTVs formed in the presence of Sar1-depleted cytosol and immunoelectron microscopy of these PCTVs using syntaxin 5 and rBet1 antibodies. PCTVs were formed in Sar1-immunodepleted cytosol and isolated. (A) The PCTVs were processed as in Fig. 1A. Intact vesicles ranging from 150 to 350 nm are seen. Bar, 500 nm. (B) The PCTVs were examined by thin section electron microscopy as in Fig. 1B. The PCTV were 300-350 nm in diameter. Bar, 200 nm. (C) Negatively stained PCTVs as in Fig. 1A were incubated with mouse anti-rBet1 (10 nm gold particles) and rabbit anti-syntaxin-5 (15 nm gold particles) antibodies. Representative views are shown in i-iii. In iv, pre-immune IgG was used. For details, see Materials and Methods.

Sar1 contained the v-SNAREs, rBet1 and syntaxin 5, on their surface, we used immunogold labeling as shown in Fig. 10C. The double-labeling technique demonstrated that both proteins were localized on these vesicles (Fig. 10Ci-iii). Control studies using pre-immune IgG showed no immuno-gold labeling (Fig. 10Civ).

To determine whether a Rab-GTPase was involved in PCTV budding, Rab-GTPase was stripped from the ER membrane by pre-incubation with GDP followed by Rab-GDI (Mukherjee et al., 2000), as confirmed by immunoblot (Fig. 5D), and stripped from the cytosol by immunodepletion (Fig. 5D). Using this Rab-depleted system, PCTV budding continued at the same rate as native ER and cytosol, but enhanced budding was not observed (Table 1). These data are consistent with our previous observations using anti-Rab1 and Rab2 antibodies that were found not to inhibit the transport of TAG from ER to Golgi (Kumar and Mansbach, 1997).

Sar1 is required for protein vesicle budding but not for lipid vesicle budding to occur

Since Sar1 depletion enhanced lipid vesicle formation, we determined whether it affected protein vesicle formation. In experiments in which both TAG and proteins were radiolabeled, when the ER was washed with urea to remove Sar1 and the cytosol immunodepleted of Sar1, we confirmed (Fig. 8C) the increase in ^{14}C-TAG output seen in Fig. 8A,B. By contrast, using native cytosol, the ^{3}H-protein radioactivity increase over background seen in Fig. 2C and confirmed in Fig. 8D (closed squares) was eliminated from the mid-portion of the gradient when Sar1 was depleted (Fig. 8D, closed circles). The findings indicate that no protein vesicle budding occurred

in the absence of Sar1. The Sar1 depletion did increase newly synthesized proteins in the low-density part of the gradient where PCTVs are found (Fig. 8D, closed circles). To examine the specificity of the Sar1 antibody, exogenous recombinant Sar1 was added to the reaction. To this end, Sar1 was immunodepleted from the cytosol; this cytosol contained neither Sar1 (Fig. 9A) nor the anti-Sar1 antibodies (data not shown). Sar1 was removed from the ER by urea washing. By using this Sar1-depleted cytosol and ER, recombinant Sar1 reduced lipid vesicle output to the levels found using native cytosol (Fig. 8E) and restored protein vesicle output (Fig. 8F). These findings confirm the specificity of the Sar1 antibodies and suggest that, similar to other systems, Sar1 is necessary for COPII-dependent protein vesicle budding in the small intestine.

To further demonstrate that the intestine is similar to other tissues with respect to the requirement of Sar1 for protein vesicle budding, we added the kinase inhibitor H89, which prevents Sar1 recruitment to ER membranes (Aridor and Balch, 2000). In both the presence and the absence of H89, ^{14}C-total dpm counts above background levels, marking pre-chylomicron-TAG, were found in the light portion of the gradient (Fig. 11A). The lack of effect of H89 on PCTV formation supports the data shown in Table 1. As expected (Table 1), GTPγS had no effect on PCTV budding (Fig. 11A). By contrast, H89 completely blocked protein vesicle formation (Fig. 11B). The addition of GTPγS reduced protein vesicle formation by 25% (Fig. 11B), consistent with the findings of other workers (Barlowe et al., 1994; Rexach and Schekman, 1991).

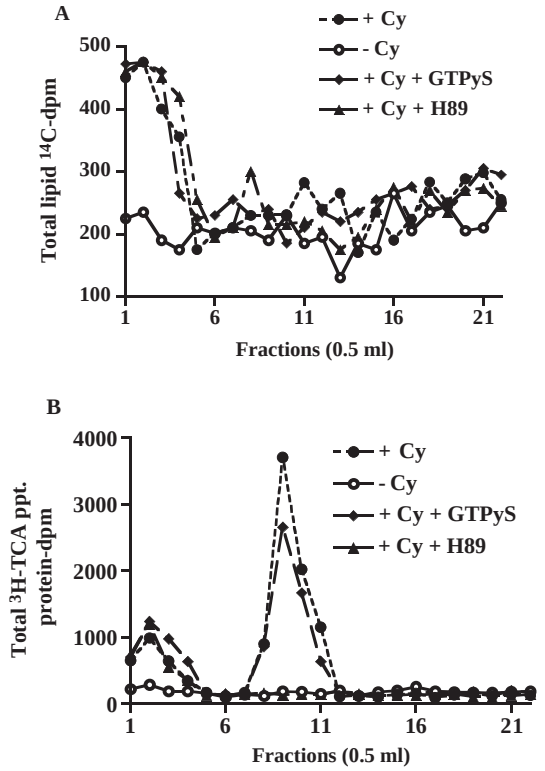

Fig. 11. The effect of GTPγS and H89 on PCTV and protein vesicle budding. 500 μg ER protein was incubated with (+) or without (–) cytosol or cytosol to which GTPγS (200 μM) or H89 (200 μM) was added as indicated in the key. (A) The total ^{14}C-TAG-dpm of each 0.5 ml fraction was determined. (B) The total ^{3}H-TCA precipitable protein-dpm was measured in each 0.5 ml fraction.

Discussion

COPII proteins mediate the cargo selection and budding of vesicles containing nascent proteins from the ER (Barlowe et al., 1994). Following the attachment of the small GTPase Sar1, Sec23/Sec24 are recruited to the membrane. By interacting with transmembrane proteins such as p58, Sec23 participates in concentrating proteins destined for export in the ER membrane (Aridor et al., 1998; Kuehn et al., 1998). Finally, budding occurs after the addition of the Sec13/Sec 31 complex. In the present study, we examined the function of COPII proteins in mediating the vesicular movement of nascent triglyceride-rich particles from the ER to the Golgi complex. Two steps were examined in detail: budding and fusion with the Golgi complex. First, we demonstrated that PCTVs could be generated from isolated ER and met the criteria of budded vesicles. The PCTVs were enriched with COPII proteins compared with the ER. However, when the function of COPII was inhibited, the ER still generated membrane-bound vesicles that had the same morphology as PCTVs. However, these lipid vesicles lacked COPII and several other accessory proteins and were unable to fuse with the Golgi complex. These findings suggest that COPII or COPII-associated proteins may be required to generate PCTVs that can fuse with the Golgi complex, but that vesicles containing TAG can bud from the ER in a COPII-independent manner.

Previous studies of COPII function have emphasized the general role of the coatomer in mediating protein export from the ER. However, recent studies have suggested that vesicles carrying specialized cargos may travel in vesicles that are distinct from those which carry the bulk of nascent proteins. For example, export of the yeast glycosylphosphatidylinositol (GPI) linked protein, Gas1p, is inhibited by conditions that do not affect the export of other nascent proteins. Thus, inhibition of ceramide synthesis, genetic deletion of either Emp24 (a potential cargo receptor) or the α-subunit of COPI, all result in decreased ER export of Gas1p but not other proteins (Schimmoler et al., 1995; Sütterlin et al., 1997). By using density centrifugation to separate vesicles budded from the ER, Gas1p and Yps1 (another GPI-linked protein) were found in a population of ER-derived vesicles that were distinct from those carrying the bulk of nascent secretory proteins (Muñiz et al., 2001). Whether budding from the ER of vesicles containing GPI-linked proteins requires COPI or COPII remains unclear. A second example is the large elongated (>300 nm) vesicles that arise from the ER and contain the rod-like structures of nascent procollagen. These are morphologically distinct from the smaller (60-80 nm) COPII coated vesicles that mediate the transport of most proteins from the ER. Further, the procollagen transport vesicles exclude ts-045-G, a marker of bulk protein movement from the ER (Stephens and Pepperkok, 2002). Another distinct cargo that moves from the ER to the Golgi complex is lipid that is transported with surrounding lipoproteins. In the intestinal mucosal cell these are known as PCTVs (Kumar and Mansbach, 1999). In the liver, pre-very low-density lipoproteins (VLDL) transport vesicles are likely to have a similar biogenesis and function as PCTVs. Little is

known about the mechanisms that regulate either the budding of these lipid-rich particles or their fusion with the Golgi complex.

The present study describes an in vitro system for examining the generation of lipid particles (PCTV) from isolated intestinal ER. The development of the system was based on in vitro techniques that have been used to examine the regulation of nascent proteins from the ER and studies of lipid trafficking from our laboratory. One important conclusion of this study is that the PCTVs we generated meet the criteria of a budded vesicle. The conclusion is supported by the following: (1) EM studies that show intact vesicles of appropriate size (350-500 nm) to contain chylomicrons (100-500 nm (Zilversmit, 1967) enclosed in a membrane bilayer; (2) inhibition by incubation at 4°C; (3) concentration of the vesicle-membrane proteins syntaxin 5 and rBet1 in the PCTVs compared with the ER; (4) protease-resistance of the chylomicron cargo protein apoB-48, indicating a membrane-enclosed vesicle; (5) exclusion of ER resident proteins, calnexin and calreticulin from PCTVs, similar to that observed by Rowe et al. in protein vesicles (Rowe et al., 1996); and (6) fusion of isolated PCTVs with the Golgi. Thus, PCTVs meet similar biochemical and functional criteria that have qualified the nascent-protein-containing COPII vesicles as physiologic carriers. A second conclusion is that PCTVs have distinct morphologic and biochemical features when compared with vesicles that export the bulk of nascent proteins as their major cargo. Thus, PCTVs are larger than protein vesicles (150-500 nm vs 60-80 nm), have an electron dense core, reflecting their lipid cargo, vs a lucent core for protein vesicles, and contain apoB-48. However, the two classes of vesicles do share some properties: both require ATP and cytosol for budding, enrich selective ER membrane proteins, and have COPII proteins on their surface.

The initial identification of Sec13, Sec24, Sec31 and Sar1 on PCTVs suggested that COPII proteins might be involved in their budding. To test the function of COPII proteins in chylomicron export from the ER we used antibodies to two proteins required for protein vesicle budding, Sar1 (Barlowe et al., 1993) and Sec31 (Tang et al., 2000). Treatment by Sar1 antibody completely blocked protein vesicle generation as would be expected for this COPII-dependent process. However, treatment by either Sar1 or Sec31 antibody increased ^{3}H-TAG export from the ER by approximately six- to tenfold. When all peripheral proteins, including Sar1 and Sec31, were removed from the ER by urea, Sar1- or Sec31-depleted cytosol continued to support PCTV generation. These unexpected results suggested that formation of PCTV might be COPII independent. To confirm this theory, we used H89, which has been shown to block COPII assembly by preventing Sar1 from attaching to the ER membrane (Aridor and Balch, 2000). H89 resulted in the same rate of lipid vesicle budding as controls not treated with H89, but not at the increased rate seen in our antibody inhibition studies. The explanation for the quantitative differences between antibodies and H89 on PCTV budding is unclear. Further, recombinant Sar1 protein completely reversed conditions in which Sar1 was depleted from the cytosol and ER confirming the specificity of the depletion experiments. Lipid vesicles generated in the absence of COPII proteins were morphologically intact with cargo protein (apoB-48) that resisted protease digestion, and continued to concentrate the membrane proteins rBet1 and syntaxin 5. These findings provide compelling evidence that vesicles containing nascent TAG can be generated by a COPII-independent mechanism. COPI coat proteins are generally associated with retrograde movement from intermediate compartments and the Golgi complex to the ER, and inter-Golgi stack movement. There are reports of COPI coat proteins mediating budding from ER (Bednarek et al., 1995; Sütterlin et al., 1997). However, the absence of COPI proteins from PCTV by immunoblot analysis makes it less likely that they mediate PCTV generation. Preliminary studies from our laboratory (S.A.S. and C.M.M., unpublished) suggest that distinct proteins are concentrated on PCTVs compared with the ER. Although the identity of these proteins is under study, it is possible that they have a role in the formation of PCTVs.

COPII proteins are usually associated with small vesicles, making the presence of COPII proteins on large PCTVs unexpected. COPII proteins have two key functions, cargo selection and generation of a vesicle. Cargo selection is mediated by the interaction of COPII proteins with the cytoplasmic domains of ER transmembrane proteins. Interactions have been described between Sec23 and di-acidic residues on these cytoplasmic extensions (Nishimura et al., 1999). Such interactions have been observed for proteins that cycle between the ER and intermediate compartments (p58) and proteins destined for export to the plasma membrane. More recently, valine at position –1 has been suggested as an export signal for transmembrane proteins as vesicle cargo from the ER (Nufer et al., 2002), further expanding the role of cytoplasmic extensions of transmembrane proteins as an export signal.

The size of vesicle may be constrained by coat proteins. Thus, the diameter of clathrin and COPII-coated vesicles are generated within a small range (60-80nm). The selective interactions between Sec13/Sec31 heterodimers might account for the uniform size of COPII vesicles that carry nascent proteins (Lederkremer et al., 2001; Matsuoka et al., 2001). However, COPII proteins have been found on large elongated (300 nm) procollagen vesicles arising from the ER (Stephens and Pepperkok, 2002). This suggests that COPII proteins might be concentrated on some populations of ER-derived vesicles without constraining their size. The findings of the present study would be consistent with this conclusion.

Although the COPII proteins do not appear to be required for selection of a lipid rich core, apoB-48 or budding of vesicles containing a lipid core, they may participate in selecting a subset of PCTV cargo. This suggestion is supported by two observations. First, when COPII proteins are excluded from PCTV by inhibiting Sar1, p58 is no longer associated with PCTVs. However, since rBet1, syntaxin 5 and apoB-48 are concentrated in PCTVs formed in the absence of COPII proteins, other mechanisms of cargo selection must remain. Second, PCTVs generated in the absence of COPII proteins are no longer competent to fuse with the Golgi complex. These findings suggest that COPII proteins or COPII-recruited proteins are required for PCTV fusion with the Golgi complex. A candidate for mediating fusion with the Golgi complex is p58, a protein that is required for recruitment of proteins (likely to be COPI) that mediate fusion of ER-derived vesicles with the Golgi complex (Tisdale et al., 1997). Based on in vitro studies, the COPII protein Sec23 mediates the enrichment of p58 in vesicles (Kappeler et al., 1997). Under conditions that

inhibit COPII assembly and attachment of the Sec23/Sec24 complex, we find that p58 is no longer concentrated in PCTVs. The speculation that p58 is required for fusion of PCTVs is supported by our studies in which PCTVs formed in native cytosol are blocked from fusion with Golgi membranes by the addition of anti-p58 antibodies (S.A.S. and C.M.M., unpublished).

To explain the large increase seen in lipid vesicle budding when secretory protein budding is inhibited, we postulate that the PCTV budding system competes for available components of the COPII system that are required for PCTV budding other than Sar1, Sec23/24 and Sec 13/31. These might include Sec12 or Sec16. In this event, the availability of the COPII protein components for PCTV budding could be rate limiting. Alternatively, COPII proteins might exhibit an inhibitory effect on the PCTV budding mechanism. Finally, the phospholipids required for protein vesicle budding to occur might be diverted to supply the PCTV membrane. In this context, work from our laboratory and others has shown that the amount of dietary phosphatidylcholine available to enterocytes is proportional to the amount of lipid that can be exported into the lymph as chylomicrons (Kumar and Mansbach, 1997; Mansbach and Arnold, 1986b; Tso et al., 1978). Further, it would appear that the enterocytes are not able to synthesize enough phosphatidylcholine from choline to support normal chylomicron output into the lymph but are able to if lyso-phosphatidylcholine is supplied (Tso et al., 1978). Nevertheless, since lipid vesicles generated in the absence of COPII proteins appear to be unable to fuse with the Golgi complex, their physiologic relevance remains questionable.

Together, the data suggest that COPII proteins are found on PCTVs and demonstrate that COPII-interacting proteins might be needed to form a lipid vesicle that can fuse with the Golgi complex. Further, the data suggest that COPII proteins can assemble on vesicles with a lipid core that are larger than the traditional COPII vesicles of 60-80nm. However, the lipid vesicles formed in the absence of COPII proteins are unable to fuse with the Golgi complex. These observations suggest that COPII-independent mechanisms can support the budding of membrane-bound lipid vesicles from the ER, and that COPII or their associated proteins, which are required for PCTV fusion with the Golgi compartment, are not directed into such vesicles.

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