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# Sec24C is required for docking the prechylomicron transport vesicle with the Golgi

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**Abstract The rate-limiting step in the transit of dietary fat across the intestinal absorptive cell is its exit from the endoplasmic reticulum (ER) in a specialized ER-to-Golgi transport vesicle, the prechylomicron transport vesicle (PCTV). PCTV bud off from the ER membranes and have unique features; they are the largest ER-derived vesicles (average diameter 250 nm), do not require GTP and COPII proteins for their formation, and utilize VAMP7 as a v-N-ethylmaleimide sensitive factor attachment protein receptor (SNARE). However, PCTV require COPII proteins for their fusion with the Golgi, suggesting a role for them in Golgi target recognition. In support of this, PCTV contained each of the five COPII proteins when docked with the Golgi. When PCTV were fused with the Golgi, the COPII proteins were present in greatly diminished amounts, indicating they had cycled back to the cytosol. Immuno-depletion of Sec31 from the cytosol did not affect PCTV-Golgi docking, but depletion of Sec23 resulted in a 25% decrease. Immuno-depletion of Sec24C caused a nearly complete cessation of PCTV docking activity, but on the addition of recombinant Sec24C, docking activity was restored. We conclude that the COPII proteins are present at docking of PCTV with the Golgi and that Sec24C is required for this event. Sec23 plays a less important role.**—Siddiqi, S., S. A. Siddiqi, and C. M. Mansbach II. **Sec24C is required for docking the prechylomicron transport vesicle with the Golgi. *J. Lipid Res.* 2010.** 51: **1093–1100.**

**Supplementary key words** lipid absorption • transport vesicles • triacylglycerol • chylomicron

The prechylomicron transport vesicle (PCTV) is an enlarged, COPII-containing vesicle that transports the developing chylomicron from the ER (endoplasmic reticulum) to the Golgi (1, 2). This largest of the lipoproteins is uniquely formed in the intestine and is the primary mechanism by which dietary fat is delivered to peripheral targeted tissues, muscle, heart, and adipose tissue. The majority of the triacylglycerol (TAG) in the chylomicron is derived from hydrolytic products of dietary lipids that are resynthesized to TAG by the intestinal ER. The newly synthesized TAG crosses the ER membrane and forms the prechylomicron in the ER lumen in a two-step process (3). The exit step of the prechylomicron from the ER is the rate-limiting step by which dietary TAG traverses the intestinal absorptive cell (4) (5). Once detached from the ER membrane, the PCTV dock and then fuse with the Golgi using the N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) complex composed of VAMP7 (the R-SNARE), syntaxin5 (the Qa-SNARE), vti1A (the Qb-SNARE), and Bet1 (the Qc-SNARE) (2).

Our interest in the molecular mechanism of the docking of PCTV with the Golgi in relation to the COPII proteins was raised by these findings: (a) Although PCTV could be generated from ER membranes in the absence of Sar1, the initiator of the COPII complex, the vesicles formed could not fuse with the Golgi (1). The vesicles were of the same size as PCTV generated using native cytosol, contained apolipoproteinB48 (apoB48), the quintessential chylomicron apolipoprotein, and VAMP7, the R-SNARE of the PCTV-Golgi SNARE complex; however, neither docking nor fusion was possible using these vesicles. b) The liver-fatty acid binding protein (L-FABP) can, in the absence of additional cytosolic components, elaborate PCTV from intestinal ER membranes (6). However, in support of the data obtained using vesicles formed in the absence of Sar1, these L-FABP formed vesicles do not contain COPII proteins and cannot fuse with the Golgi. In

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Abbreviations: ApoB48, apolipoprotein B-48; ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; ER exit sites, ERES; L-FABP, liver fatty acid binding protein; PCTV, prechylomicron transport vesicle; SNARE, N-ethylmaleimide sensitive factor attachment protein receptor; TAG, triacylglycerol; VAMP7, vesicle associated membrane protein 7.

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sum, the data strongly suggested to us that COPII proteins played a role in the docking process with the Golgi.

Vesicles that take newly synthesized proteins from the ER to the Golgi are well described and require COPII proteins for their generation from ER membranes. These COPII proteins have been divided into an inner coat containing Sar1 and Sec23/24, and an outer coat containing Sec13/31 (7). In this construct, Sar1, in its GTP bound form, is recruited to the ER membrane and subsequently recruits Sec23/24 and then Sec13/31. These events occur in the presence of potential cargo for the vesicle at specific ER exit sites (ERES) (8–10). The completed structure then deforms the ER membrane and, in a fission step, is detached from the membrane. Most of these vesicles are remarkably uniform in size, ~60 nm (11). Their size is constrained by the heterodimer Sec13/31, which forms a cage around the vesicle. However, new data from the Balch laboratory (12) suggest that the angles of the cage components can be altered to some degree to allow for enlarging the cage to encompass larger cargos, up to 100 nm (12). This is still not large enough to enable a chylomicron of 250 nm to be enclosed within the cage structure. By contrast, PCTV are large enough to contain chylomicrons and have been measured up to 350 nm (1).

The total data suggest that the COPII proteins are used by PCTV not as a vesicle generation mechanism but rather as a required Golgi targeting mechanism in addition to the R-SNARE VAMP7. As part of the targeting mechanism, the COPII proteins may not uncoat prior to the docking event of the PCTV with the Golgi. This report tests this hypothesis that has received support from recent data using COPII-dependent, protein vesicles (13).

## MATERIALS AND METHODS

### Materials

$^{3}$H-Oleic acid (9.2 Ci/mM) was procured from Perkin Elmer Life Sciences. Immunoblot reagents were purchased from Bio-Rad. Enhanced chemiluminescence (ECL) reagents were procured from GE Healthcare. Protease inhibitor cocktail tablets were obtained from Roche Applied Science. Other biochemicals used were analytical grade and purchased from Sigma (Sigma Chemical Co., St. Louis, MO) or local companies. Male Sprague Dawley rats, 150–200 g were purchased from Harlan (Indianapolis, IN) and fed rat chow ad lib including the night prior to sacrifice. All research was done in accordance with NIH policies and was approved by the University of Tennessee Health Science Center Institutional Animal Use and Care Committee.

### Antibodies and protein

Rabbit anti-Sar1 antibodies were raised commercially (Protein Tech Group, Chicago, IL) using recombinant Sar1 protein (Siddiqi et al., 2003). Polyclonal antibodies against rat VAMP7 were raised in rabbits against amino acids 105-123 of rat VAMP7 (14). Affinity-purified rabbit polyclonal anti-Sec24C antibodies were a gift of J.P. Paccaud (University of Geneva, Geneva, Switzerland). Rabbit polyclonal antibodies against Sec13 and Sec31 were a generous gift of Dr. F. S. Gorelick (Yale University, New Haven, CT). Goat anti-Sec 23 antibodies and rabbit polyclonal antibody to syntaxin 5 were procured from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies to rBet1 were procured from Stressgen (Vancouver, Canada). Mouse anti-vti1a monoclonal antibodies were purchased from BD Biosciences. Rabbit polyclonal anti-apolipoprotein AI (apoAI) antibodies were a gift of Dr. Patrick Tso (University of Cincinnati, Cincinnati, OH). Sec24C recombinant protein was purchased from Novus Biologicals, Inc. (Littleton, CO). Goat anti-rabbit IgG conjugated with agarose beads was purchased from Sigma. Goat anti-rabbit IgG and goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) were procured from Sigma.

### Preparation of ER, Golgi, cytosol, and labeling of enterocytes

Enterocytes from the proximal half of male Sprague Dawley rat small intestine were isolated and radiolabeled with $^{3}$H-oleate as described (15). In brief, enterocytes were isolated from intestinal villi, collected, incubated with albumin bound $^{3}$H-oleate for 30 min at 35°C and washed twice with PBS containing 2% BSA to remove the excess $^{3}$H-oleate. The labeled enterocytes were homogenized using a Parr bomb and the ER was isolated using a sucrose step gradient that was repeated to purify the ER. The purified ER contained calnexin and calreticulin, markers proteins for ER, but neither GOS28 nor rab11, markers for Golgi and endosomes, respectively (1, 14). The Golgi was isolated from non-radiolabeled enterocytes as was cytosol (1, 16). Marker proteins assessed the purity of Golgi.

### Depletion of specific COPII proteins from the cytosol and ER

Depletion of specific COPII proteins from intestinal cytosol was accomplished by incubating cytosol (1 mg protein) with 10μl of specific primary antibody at 4°C for 4 h. The antibody bound COPII proteins and any excess antibody were removed by incubation with anti-goat IgG bound to agarose beads or with anti-rabbit IgG bound to agarose beads at 4°C overnight and the beads removed by centrifugation. Depletion required two or three rounds of antibody treatment. Depletion was confirmed by immunoblot using specific antibodies. Depletion of specific ER proteins was accomplished by washing the ER membranes with 2M urea that was confirmed by immunoblot. In all studies using a specific COPII protein(s)-depleted system, both cytosol and ER were depleted.

### In vitro PCTV formation

PCTV containing $^{3}$H -TAG was formed from $^{3}$H-TAG loaded intestinal ER (1)). In brief, ER containing $^{3}$H -TAG (500 μg protein) was incubated at 37°C for 30 min with cytosol (1 mg protein) and an ATP-regenerating system in the absence of Golgi acceptor (total volume 500 μl). The incubation mixture was resolved on a continuous sucrose density gradient (0.1–1.15 M sucrose) and PCTV isolated from the light portions of the gradient. PCTV thus formed were concentrated using a Centricon-10 filter (Millipore Corp., Bedford, MA).

Specific COPII protein depleted PCTV were generated by using specific COPII protein depleted cytosol and ER. For example, Sec23- or Sec24C-depleted PCTV were generated from Sec23- or Sec24C-depleted cytosol and ER.

### In vitro docking of PCTV with Golgi

PCTV (150 μg protein) containing $^{3}$H-TAG were incubated with Golgi membranes (300 μg protein) and 500 μg cytosolic protein with 30 mM HEPES buffer containing 0.25M sucrose, 30 mM KCl, 5 mM $CaCl_2$, 1 mM EDTA and 2 mM DTT for 1 h at 4°C (total volume 500 μl). The incubation mixture was resolved on a sucrose step gradient, and the Golgi fraction obtained by aspira-



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tion (14). $^{3}$H-TAG was extracted (17) from the Golgi, and the radioactivity determined in a liquid scintillation spectrometer.

To determine the effect of Sec23, Sec24C, or Sec31 depletion on PCTV-Golgi docking, Sec23-, Sec24C-, or Sec31-depleted cytosol and Sec23-, Sec24C-, or Sec31-depleted PCTV were used in the docking assay. These data were compared with PCTV docking activity after immuno-depletion with IgG. When recombinant Sec24C (rSec24C) was added back to Sec24C immuno-depleted cytosol for purposes of restoring docking activity, the rSec24C (7.25, 14.5, or 29 μg protein as indicated) was incubated with Sec24C immuno-depleted cytosol and Sec24C-depleted PCTV for 30 min at 4°C prior to starting the docking assay. The repleted cytosol and PCTV were used in the docking assay with Golgi membranes as described above.

When anti-Sec24C antibodies were used in PCTV-Golgi docking experiments, native cytosol was used to generate PCTV. The native PCTV were incubated with anti-Sec24C antibodies (20 μl) for 40 min at 4°C prior to docking. The antibody-treated PCTV were then used in the docking assay with native cytosol and Golgi membranes.

### In vitro PCTV fusion with the Golgi

PCTV fusion with the Golgi was performed by incubating $^{3}$H-TAG loaded-PCTV (150 μg protein) with Golgi membranes (300 μg protein) and native cytosol (500 μg protein) for 30 min at 35°C with an ATP regenerating system, 5 mM $MgCl_2$, 0.25M sucrose, 30 mM HEPES, pH 7.2, 30 mM KCl, 5 mM $CaCl_2$, and 2 mM DTT; total volume, 500μl (2). Postincubation, the Golgi membranes were separated from un-reacted PCTV by a sucrose step gradient, and the Golgi-associated TAG radioactivity was determined (2).

### Isolation of PCTV-Golgi SNARE complex

Isolation of the SNARE complex was accomplished by solubilizing PCTV docked with Golgi (200 μg) using 2% Triton X-100 in PBS as described (2). In brief, the solubilized proteins were incubated overnight at 4°C with anti-rabbit VAMP7 antibody bound to anti-rabbit IgG conjugated with agarose beads. The beads were collected by centrifugation, washed eight times with PBS to remove unbound proteins/antibodies, and resuspended in Laemmle's buffer for immunoblot analysis. The beads were either boiled or not boiled in Laemmle's buffer as indicated. The immuno-precipitated proteins were separated by SDS-PAGE, then transblotted onto nitrocellulose membranes (Bio-Rad). The same membrane was probed with antibodies to VAMP7, syntaxin 5, vti1a, and rBet1.

In experiments where the ability of rSec24C to reestablish Sec24C depleted PCTV-Golgi docking was tested, 29 μg rSec24C were incubated with Sec24C depleted-PCTV (150 μg prot) and Sec24C depleted-cytosol (500 μg prot) for 60 min at 4°C. The Sec24C repleted PCTV were then incubated with Golgi membranes (300 μg prot) as for docking and the SNARE complex isolated as described (2).

### SDS-PAGE and immunoblots

Proteins were separated by SDS-PAGE, then transblotted onto nitrocellulose membranes (Bio-Rad) (1). After incubation with specific primary antibodies and peroxidase-conjugated secondary antibodies, proteins were detected by ECL (Amersham Biosciences) and Biomax film (Eastman Kodak, Rochester, NY).

### Measurement of TAG radioactivity

TAG radioactivity was determined by liquid scintillation spectroscopy (15).

## RESULTS

### COPII proteins are present at PCTV docking with the Golgi

In contrast to older information (18), more recent data would suggest that ER-to-Golgi transport vesicles which contain COPII proteins are attached to Golgi tethering proteins prior to their eventual fusion with the Golgi (13). We wished to determine if that were true for PCTV and Golgi membranes in the intestine. To this end, we incubated PCTV, which contain all the COPII proteins (**Fig. 1A**, lane 1) with Golgi under conditions that favor docking (2). Although the Golgi gave a weak signal for each of the COPII proteins prior to incubation (Fig. 1A, lane 2), after a 60 min incubation with PCTV, the COPII proteins in the Golgi gave a much stronger signal (Fig. 1A, lane 3). One interpretation of these data is that the COPII proteins on PCTV do not un-coat prior to docking with the Golgi and thus remain during the docking process resulting in their increased concentration on isolation of the Golgi. Under conditions in which fusion with the Golgi would be expected (2), the signals for all the COPII proteins were greatly diminished (Fig. 1A, lane 4), suggesting that the COPII proteins returned to cytosol after fusion. To establish that PCTV were fused with the Golgi, we took advantage of our prior observation that prechylomicrons in PCTV do not contain apoAI (1). However, apoAI is present in the Golgi lumen (1, 2) and becomes associated with the prechylomicrons when they enter the Golgi lumen after PCTV-Golgi fusion (1, 2). In the current experiments, as before, prechylomicrons in PCTV contain no apoAI (Fig. 1B, chylo), and the few chylomicrons isolated from

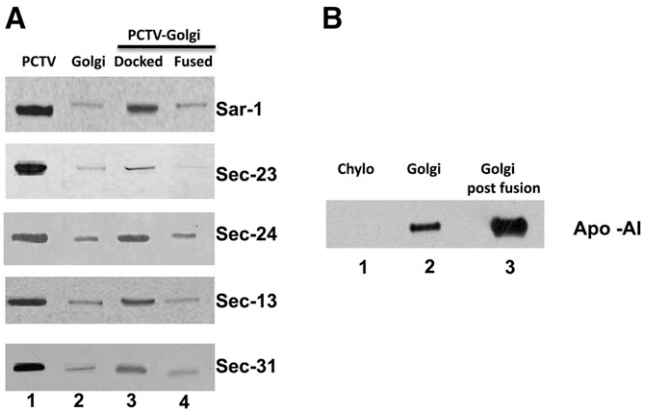


**Fig. 1.** The effect of docking and fusion of PCTV with Golgi membranes on COPII proteins and chylomicron acquisition of apoAI on fusion with the Golgi. A: Equal amounts of proteins (40 μg) were separated by 12% SDS-PAGE, transblotted to nitrocellulose membranes, and the proteins detected by ECL using specific antibodies to the COPII proteins as indicated. The presence of the COPII proteins was detected in PCTV (lane 1), Golgi (lane 2), PCTV docked with the Golgi (lane 3), and PCTV fused with the Golgi (lane 4). Methods for docking and fusion of PCTV with the Golgi are described in "Materials and Methods." B: Chylomicrons isolated from PCTV (lane 1), Golgi prior to fusion with PCTV (lane 2), and Golgi after fusion with PCTV (lane 3) were obtained and their proteins separated by SDS-PAGE. After transblotting, apoAI was identified by ECL using specific antibodies. ECL, enhanced chemiluminescence; PCTV, prechylomicron transport vesicle.



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the Golgi lumen by carbonate treatment show small amounts of apoAI (Fig. 1B, Golgi). By contrast, under conditions where fusion of the PCTV with the Golgi is expected, the Golgi lumen becomes enriched with chylomicrons and the amount of apoAI associated with them is greatly (3-fold) increased (Fig. 1B, Golgi post fusion), supporting our prior data (1, 2).

### Immunodepletion of Sec23 and its effect on PCTV-Golgi docking

Our next aim was to determine which of the COPII proteins was primarily important for target recognition for PCTV with the Golgi. We have previously shown that in the absence of Sar1, PCTV do not fuse with the Golgi (1, 6). We next considered Sec23. As expected, intestinal cytosol contained significant amounts of Sec23 as shown by its strong signal on immunoblot (**Fig. 2A**, lane 1). By contrast, using bead-bound specific anti-Sec23 antibodies, Sec 23 was completely removed from the cytosol by the immunodepletion (Fig. 2A, lane 2). PCTV generated in IgG-depleted cytosol contained Sec23 (Fig. 2A, lane 3), whereas PCTV formed in Sec23-depleted cytosol did not (Fig. 2A, lane 4), as expected. Sec24C was modestly reduced by the immunodepletion of Sec23 (Fig. 2A, Sec24C).

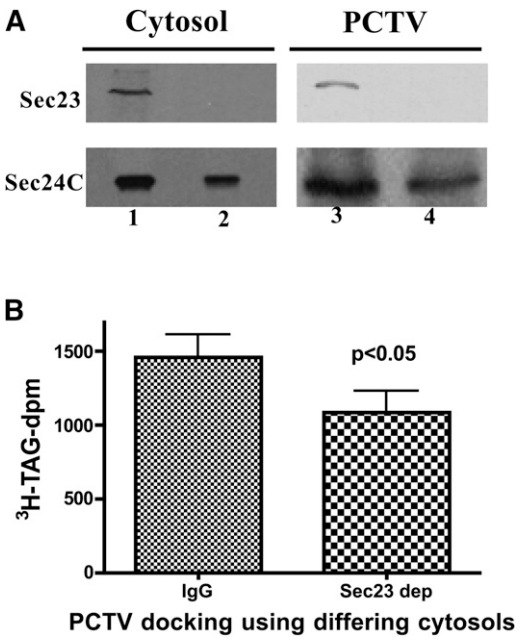


**Fig. 2.** The effect of Sec23 depletion from the cytosol and ER on PCTV docking with Golgi membranes. A: Sec 23 was immunodepleted from native rat cytosol using specific antibodies. 40 μg of cytosolic protein was separated by SDS-PAGE and Sec23 or Sec24C identified by ECL. Cytosol was immunodepleted by IgG or anti-Sec 23 antibodies as indicated. PCTV were generated using IgG depleted or Sec23 immunodepleted cytosol and its proteins separated by SDS-PAGE. Sec23 or Sec24C was identified by immunoblot using anti-Sec23 or Sec24C antibodies as shown. B: PCTV (150 μg protein) were generated using IgG or Sec23 immunodepleted cytosol and were docked with Golgi membranes (300 μg protein). The $^3$H-TAG-dpm associated with the Golgi was determined in each case (ordinate). The *P* value tests the differences between the means ± 1 SEM, N = 4. ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; PCTV, prechylomicron transport vesicle; TAG, triacylglycerol.

When $^3$H-TAG loaded PCTV generated in IgG immunodepleted cytosol were incubated with Golgi membranes under conditions favoring docking (2), a robust $^3$H-TAG signal was obtained in the Golgi fraction, indicating significant amounts of docking (Fig. 2B, IgG). However, when PCTV generated in Sec23-depleted cytosol were incubated with Golgi under conditions favoring docking, a 25% reduction in the ability of the Sec23-depleted PCTV to dock with the Golgi was found (Fig. 2A, Sec23 dep). The reduced docking activity of the Sec23-depleted cytosol may be due to partial Sec24C reduction as a result of the Sec23 depletion (Fig. 2A, Sec24C). An interpretation of these data is that Sec23, while important as a Golgi targeting signal, is not required for docking to occur.

### Immunodepletion of Sec24C and its effect on PCTV-Golgi docking

Because immuno-depletion of Sec23 seemed to have only a modest effect on PCTV-Golgi docking, we immunodepleted Sec24C from cytosol using specific antibodies. This resulted in the removal of Sec24C from the cytosol (**Fig. 3A**, compare IgG depleted cytosol with Sec24C depleted cytosol). Densitometric analysis revealed that some (73%) Sec23 remained (Fig. 3A, Sec24C depl), presumably because only Sec24C was removed, leaving Sec23 potentially complexed to the remaining Sec24 paralogs (Sec24A, B, or D). When the Sec24C-depleted cytosol was used in place of IgG-depleted cytosol in the generation of PCTV from Sec24C-depleted ER membranes, the PCTV produced were also devoid of Sec24C, as expected (Fig. 3A, compare PCTV formed in IgG- depleted cytosol with PCTV formed in Sec24C-depleted cytosol). Importantly, the modest reduction in Sec31 seen in the cytosol in the Sec24C-depleted cytosol was not reflected in the intensity of the Sec31 signal on Sec24C-depleted PCTV (Fig. 3, lane 4).

When $^3$H-TAG loaded Sec24C-depleted PCTV were tested for their ability to fuse with the Golgi, they were unable to do so (Fig. 3B, Dep), whereas PCTV generated using IgG-depleted cytosol gave a robust $^3$H-TAG signal in the Golgi (Fig. 3B, IgG). These data highlight the important role played by Sec24C in PCTV target recognition. An alternative explanation of the data is that the anti-Sec24C antibodies removed not only Sec24C but one or more other unknown proteins that are important for Golgi docking and that the effect seen on immuno-depletion is due to the lack of this protein(s) rather than the lack of Sec24C. To address this question, we repleted Sec24C-depleted cytosol with increasing amounts of recombinant Sec24C (Fig. 3B). After adding 7.25 μg Sec24C, no increase in docking was seen. However, as the amount of Sec24C was increased, PCTV docking with the Golgi progressively increased, reaching 74% the activity of IgG-depleted cytosol when 29 μg Sec24C was added (Fig. 3B) (29). As measured by semi-quantitative immunoblot, cytosol contains 1.2 μg Sec 24C/mg prot. The excess rSec24C required to be added to restore nearly full docking activity presumably relates to the GST tag on the recombinant



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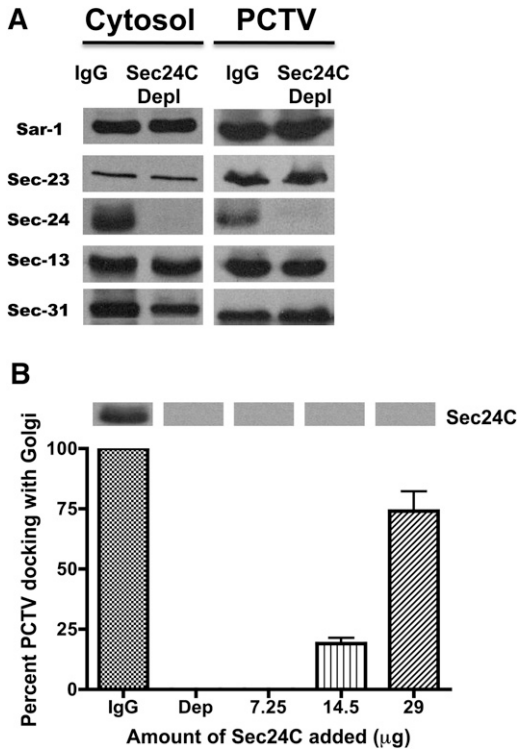


**Fig. 3.** Depletion of Sec24C from cytosol and ER impairs PCTV-Golgi docking. A: The effect of Sec24C immuno-depletion of cytosol on cytosolic COPII proteins. Cytosol: Rat intestinal cytosol was either immunodepleted with IgG (IgG) or anti-Sec24C antibodies (Sec24C Depl) as indicated and the cytosol probed for the COPII proteins using specific antibodies. 40 μg cytosolic protein was separated by SDS-PAGE and the COPII proteins identified using ECL after transblotting. The Sec23 band density of cytosol was quantitated using the Gel Doc XR Imaging System (BioRad). PCTV: PCTV (40 μg prot) formed using either IgG (IgG) or Sec24C immunodepleted (Sec24C Depl) cytosol was immunoblotted for the COPII proteins including Sec24C after separation by SDS-PAGE and the proteins identified by ECL after transblotting. B: The effect of Sec24C immunodepleted cytosol on PCTV docking with the Golgi and the effect on docking activity of adding recombinant Sec24C to Sec24C depleted cytosol. PCTV were generated in either IgG depleted (IgG) or Sec24C depleted rat intestinal cytosol to which either none (Dep) or the indicated amount of rSec24C had been added. The Sec24C status of the cytosols prior to the addition of rSec24C is shown above the graph in immunoblots. In each case, the $^{3}$H-TAG loaded PCTV were docked with intestinal Golgi and the $^{3}$H-TAG-dpm associated with the Golgi determined. The dpm (2946 $^{3}$H-TAG-dpm) found for PCTV formed in IgG depleted cytosol was set to 100%. The data are the percentage of $^{3}$H-TAG dpm associated with the Golgi by comparison to the dpm of IgG depleted cytosol. The data are the mean values ± 1 SEM, N = 4. ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; PCTV, prechylomicron transport vesicle; TAG, triacylglycerol.

protein, potential glycosylation changes in the recombinant as compared with the native protein (19), and the structural integrity of the rSec24C. These data show the importance of Sec24C on PCTV as a contributory Golgi-targeting mechanism with the R-SNARE VAMP7 (2).

### Immunodepletion of Sec31 and its effect on PCTV-Golgi docking

We considered the possibility that Sec31 could also have an effect on PCTV docking. To address this possibility, we immuno-depleted cytosol of Sec31 (data not shown) and used this cytosol to bud $^{3}$H-TAG loaded PCTV from ER membranes. By contrast to the data obtained with Sec23 and Sec24C immuno-depletion, on Sec31 removal, only a modest, nonsignificant reduction was seen (**Fig. 4**).

### rSec24C mediates PCTV-Golgi SNARE complex formation

We next wished to determine if the rSec24C added to Sec24C immuno-depleted cytosol would not only promote docking of PCTV with the Golgi but also would enable the formation of the PCTV-Golgi SNARE complex preparatory to fusion. When Sec24C-depleted cytosol and PCTV were incubated with Golgi membranes under conditions where docking would be expected (see "Materials and Methods"), no SNARE proteins for the PCTV-Golgi SNARE complex at 112 kDa were found (**Fig. 5**, lane 1), nor was any SNARE complex was found on boiling (Fig. 5, lane 2). By contrast, when rSec24C, 29 μg, was incubated with Sec24C-depleted PCTV prior to docking with the Golgi using Sec24C-depleted cytosol (2), a 112 kDa complex was formed (Fig. 5, lane 3). This complex consisted of syntaxin5, VAMP7, vti1a, and, rBet1 (Fig. 5, lane 3) when the sample was not boiled and DTT was not added. These data, which support our prior studies (2), suggest that the rSec24C was effective in bringing the PCTV close enough to the Golgi to allow for SNARE complex formation. By contrast to the complex formed when the proteins were separated under mild conditions, when the proteins were separated after boiling in Laemmle's buffer, the SNARE complex disassociated into its monomer components as shown for each of the components (Fig. 5, lane 4). If the $M_r$ of the monomer species were added together, the expected $M_r$ is 113 kDa, close to the $M_r$ of 112 kDa as shown in Fig. 5 for the SNARE complex. Contamination by ER membranes potentially providing proteins important for PCTV docking is unlikely because neither the cytosol nor

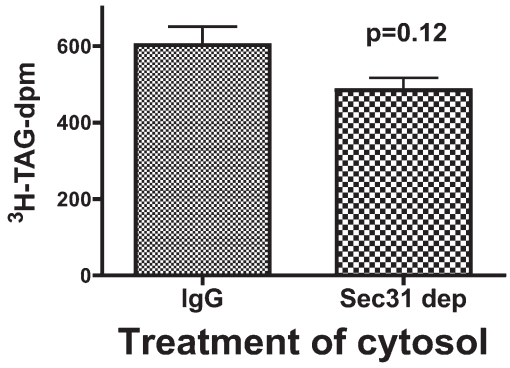


**Fig. 4.** The effect of Sec31 immunodepletion on PCTV docking with intestinal Golgi. Cytosol immunodepleted of Sec31 (data not shown) using specific anti-Sec31 antibodies or IgG was used to generate PCTV. PCTV (150 μg protein) formed using these cytosols were docked with intestinal Golgi (300 μg protein) as described in "Materials and Methods." After incubation, the number of $^{3}$H-TAG-dpm associated with the Golgi was determined. IgG indicates PCTV formed in IgG depleted cytosol and Sec31 dep indicates Sec31 depleted cytosol. The data are the means ± 1 SEM, N = 4. PCTV, prechylomicron transport vesicle; TAG, triacylglycerol.



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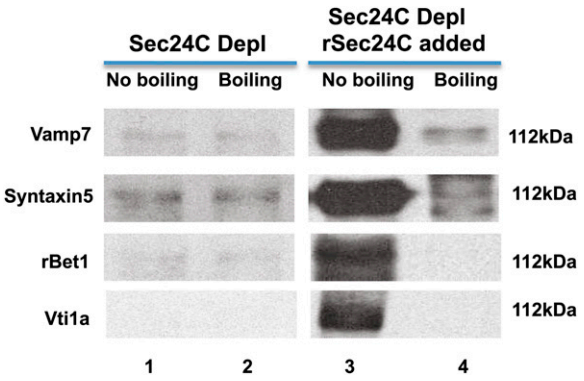


**Fig. 5.** The effect of Sec24C immuno-depletion on the formation of the PCTV-Golgi SNARE complex. PCTV were generated using Sec24C depleted cytosol and ER (Sec24C Depl, lanes 1 and 2) or PCTV generated in Sec24C depleted cytosol and ER followed by the incubation of the PCTV with rSec24C (29 μg) (Sec24C Depl, rSec24C added, lanes 3 and 4). The PCTV were then docked with the Golgi. Post docking, the Golgi membranes were isolated, solubilized in 2% Triton X-100, and the proteins immuno-precipitated using bead-bound anti-VAMP7 antibodies. The beads were not boiled or boiled in Laemmli's buffer as indicated. The proteins were separated by SDS-PAGE, transblotted, and the transblots probed using specific antibodies to the expected SNARE proteins (2). The blots shown are at 112 kDa, the Mr of the PCTV-Golgi SNARE complex (2). On boiling, the SNARE proteins disassociated from the complex and moved at their monomer $M_r$ (data not shown). ER, endoplasmic reticulum; PCTV, prechylomicron transport vesicle.

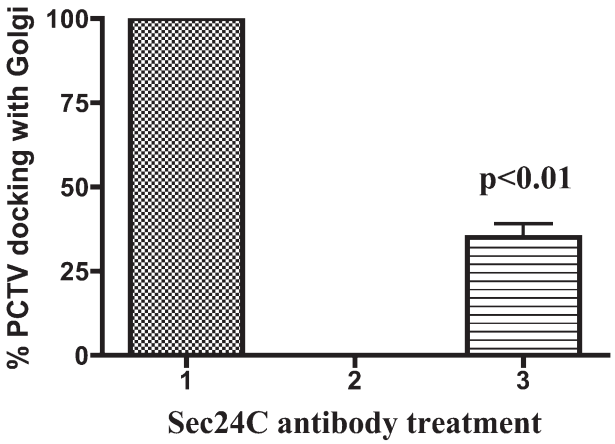


**Fig. 6.** Sec24C antibody treatment of PCTV blocks it's docking with Golgi membranes. $^3$H-TAG loaded PCTV were generated using native cytosol and ER and docked with Golgi membranes. The $^3$H-dpm found associated with the Golgi under these conditions was set to 100% (bar1). In bar 2, cytosol and ER immunodepleted of Sec24C was used to generate PCTV devoid of Sec24C that was then incubated in Sec24C depleted cytosol with Golgi membranes. The percentage of $^3$H-dpm found docked with the Golgi under these conditions as compared with when native PCTV was used is shown. In bar 3, PCTV formed using native cytosol and ER were treated with 20 μl of anti-Sec24C antibody at 4°C for 30 min and then incubated with native cytosol and Golgi in a docking assay. The percentage of $^3$H-TAG-dpm associated with the Golgi under these conditions as compared with the results obtained in bar 1 are shown. The *P* values test the difference between the means for bar 1 and bar 3. The data are the mean ± SEM, N = 4. PCTV, prechylomicron transport vesicle; TAG, triacylglycerol.

PCTV used contain the ER marker protein calnexin under the conditions of our experiments (1, 14), and PCTV are significantly less dense than ER (14).

It is possible that Sec24C removal by immuno-precipitation in some way changes either the structure or protein composition of PCTV that affects their docking to the Golgi by an alternate means other than the absence of Sec24C. To address this question, we incubated PCTV with anti-Sec24C antibodies under various conditions (**Fig. 6**). IgG-treated intestinal cytosol and PCTV generated from it were incubated with Golgi membranes and the amount of docking determined (Fig. 6, bar 1). The docking activity under these conditions compared with when no cytosol was included in the incubation was set to 100%. By contrast, when Sec24C-depleted cytosol and PCTV were incubated with Golgi membranes (Fig. 6, bar 2), essentially no PCTV were associated with the Golgi, confirming the data shown in Fig. 3. Consistent with these data, when PCTV were incubated with anti-Sec24C antibodies prior to its incubation with Golgi membranes, docking activity was reduced to 35% of the activity found under native conditions (Fig. 6, bar 3). The incomplete antibody blockade could be due to incomplete blocking of antigenic sites on Sec24C or an inefficient blockade by the antibody, or recruitment of Sec24C from the native cytosol to the PCTV.

## DISCUSSION

COPII proteins are quintessential components of ER-to-Golgi transport vesicles. Without them, no anterograde transport occurs for newly synthesized proteins or other potential cargoes that require export from the ER to the Golgi. In humans, multiple mutations have been described that both affect this process and highlight the importance of its various components (20, 21). For the present report, the most pertinent of the mutations is Chylomicron retention disease (CRD) in which Sar1b is mutated in the GTP binding pocket, making the Sar1 component of the COPII proteins inoperative (22). Also relevant to the present data, cranio-lenticulo-sutural dysplasia (CLSD) has been found due to a F382L-SEC23A mutation, proposed to be caused by a reduced affinity of the Sec13/31 COPII component for the mutated Sec23A (23). In those tissues affected by the mutation, it was found that expression of the potentially compensating SEC23B paralog of SEC23A was low, leading to the effective loss of Sec23 function (23). In sum, these data support the importance of COPII proteins in ER-to-Golgi vesicle trafficking. The data in humans are supported by a variety of in vitro studies using mutated recombinant proteins.

In the iconic model of COPII protein–dependant vesicle formation, the process of vesicle cargo selection, membrane deformation, and fission is initiated by the exchange of Sar1-GDP for GTP mediated by Sec12 at ERES. This concept is supported by studies that show reduction in vesicle formation in the presence of Sar1 mutated such that it was unable to be inserted in the ER membrane (24) or had a reduced affinity for GTP (25). However, more recent work has shown that the GDP-GTP exchange oc-



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curs rapidly and repetitively but is primarily maintained in the GTP form by Sec12. This elongates the time COPII proteins remain on the ER membrane, perhaps allowing adequate time for cargo binding (26). In addition, cargo binding and v-SNARE binding to Sec23/24 (26) maintain coat stability. Ultimately, for fission of the vesicle to occur, Sar1-GTP hydrolysis is required; without it, vesicles accumulate on the ER membrane (27). Even after fission from the ERES and final Sar1 GTP hydrolysis with its resultant detachment from the membrane, Sec23/24 remains on the vesicle (28), an attachment aided by cargo (8) and V-SNARE binding (26).

In the case of PCTV, however, a different paradigm is proposed. We have previously shown that PCTV budding does not require GTP (1), and therefore, no GTP loading of Sar1 occurs. Since the deployment of the N-terminal α-helix of Sar1, which inserts into the ER membrane, is GTP dependant (11, 24), we suggest that Sar1, shown to exist on PCTV (1), is attached to ERES for PCTV by a different mechanism, such as binding to the cytosolic domain(s) of cargo protein(s) (29) and/or Sec23/24 (11). When recombinant L-FABP (rL-FABP) is used to form PCTV, no Sar1is present on the vesicles even when they are later incubated with native cytosol, replete with Sar1 (6). These data support the speculation that Sar1 attachment to PCTV occurs contemporaneously with PCTV formation on the ER. Further, in unpublished work, we have shown that Sar1 is immuno-precipitated from Triton X-100 solubilized ER membranes using anti-apoB48, anti-L-FABP, anti-Sec24C, and anti-VAMP7 antibodies. We have shown that each of these proteins plays a role in PCTV generation (1, 2, 6, 14). The heterodimer Sec13/31 is also present on PCTV (1).

In COPII-dependent vesicles, Sec 13/31 has been proposed to form a cage around the vesicle whose diameter can be varied to encompass vesicles 60–100 nm (12). PCTV are much larger, averaging 250 nm (1). While it is possible that a Sec13/31 cage may surround PCTV, it is also possible that it does not but rather is present as a single or double heterodimer. This hypothesis is supported by data showing that PCTV can be budded from ER membranes in the absence of Sec31 but in the presence of Sar1 and Sec23/24 (1), as compared with the proposal of Stagg et al. that Sec13/31 acts to deform the ER membrane, promoting fission of the budding vesicle (12) and is required for vesicle budding (30).

The data presented in this report extend our prior observation that, in the absence of the COPII proteins, PCTV do not dock or fuse with the Golgi (1) by showing that in contrast to COPII vesicles, all of the COPII proteins are present at docking of PCTV with the Golgi. Also unlike protein vesicles, PCTV may be required to maintain COPII proteins as part of their targeting mechanism with the Golgi. In support of this idea, Sfb2p, the yeast homolog of Sec 23, binds to Sed5p, the yeast homolog of syntaxin5 (31). Syntaxin5 is the Qa SNARE for PCTV (2). In addition, Sec23 has been shown to be important for tethering ER transport vesicles to the TRAPP1 protein, Bet3 (13). The current studies extend this observation by showing that all of the COPII proteins on PCTV remain not only at the tethering step but at the docking step as well. On fusion with the Golgi, the COPII proteins are found in greatly diminished amount on the Golgi membranes, presumably because they have returned to the ER for additional rounds of COPII vesicle or PCTV transport.

The SAR1 gene has two paralogs, SAR1A and SAR1B, which yield the expression of proteins that vary by 20 amino acids. While mutations in SAR1A have not been reported because they may be lethal, mutations in SAR1B have (22, 32). Although the mutations and truncations of the SAR1B gene vary, the phenotype resulting from these genetic abnormalities yields the inability to mount a chylomicronemia after a fatty meal. If PCTV are produced in the absence of Sar1 in vitro (1), why should the mutated Sar1b protein lead to the absence of chylomicronemia in vivo (32)? One possibility is that the PCTV that are produced in the absence of Sar1 do not fuse with the Golgi, resulting in their retention in the cytosol. These immature PCTV would then sequester proteins important for PCTV generation on PCTV retained in the cytosol, making them unavailable for recycling back to the ER to generate another round of PCTV. The return of these proteins to the ER may control PCTV generation in the native state, supporting the observation that chylomicron exit from the ER is the rate-limiting step in the transcellular movement of neutral lipids (5). Further, with respect to the current observations, in the absence of Sar1, neither Sec24C nor Sec31 are present on the PCTV produced under these conditions (1). Thus the potential binding sites on Sec24C for PCTV cargo proteins such as apoB48 (33) are not enough to enable Sec24C binding to PCTV. The prior binding of Sar1 is required.

Our data also supports prior data that COPII proteins can be recruited back onto stripped vesicles, which enable fusion (13). Here, PCTV generated in the absence of Sec24C were unable to fuse with the Golgi. By contrast, when the PCTV were incubated with rSec24C, the PCTV readily fused with the Golgi.

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