SNARE Status Regulates Tether Recruitment and Function in Homotypic COPII Vesicle Fusion*

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In mammals, coat complex II (COPII)-coated transport vesicles deliver secretory cargo to vesicular tubular clusters (VTCs) that facilitate cargo sorting and transport to the Golgi. We documented in vitro tethering and SNARE-dependent homotypic fusion of endoplasmic reticulum-derived COPII transport vesicles to form larger cargo containers characteristic of VTCs (Xu, D., and Hay, J. C. (2004) J. Cell Biol. 167, 997–1003). COPII vesicles thus appear to contain all necessary components for homotypic tethering and fusion, providing a pathway for de novo VTC biogenesis. Here we demonstrate that antibodies against the endoplasmic reticulum/Golgi SNARE Syntaxin 5 inhibit COPII vesicle homotypic tethering as well as fusion, implying an unanticipated role for SNAREs upstream of fusion. Inhibition of SNARE complex access and/or disassembly with dominant-negative α-soluble NSF attachment protein (SNAP) also inhibited tethering, implicating SNARE status as a critical determinant in COPII vesicle tethering. The tethering-defective vesicles generated in the presence of dominant-negative α-SNAP specifically lacked the Rab1 effectors p115 and GM130 but not other peripheral membrane proteins. Furthermore, Rab effectors, including p115, were shown to be required for homotypic COPII vesicle tethering. Thus, our results demonstrate a requirement for SNARE-dependent tether recruitment and function in COPII vesicle fusion. We anticipate that recruitment of tether molecules by an upstream SNARE signal ensures that tethering events are initiated only at focal sites containing appropriately poised fusion machinery.

Vesicle targeting and fusion in the endomembrane system are mediated by several conserved protein families. Rab GTPases bind vesicles and activate and/or recruit effectors for several processes upstream of fusion, including the proper formation or loading of vesicles (1–3) and the activation and recruitment of the transport step-specific tethering machinery that mediates the initial pairing of a vesicle and its target membrane (4). Tethers are thought to form a physical link between two membranes prior to SNARE4 engagement, impart specificity to membrane fusion reactions, and create a permissive condition for SNARE assembly and fusion. Some of the defining characteristics of tethers appear to be as follows: 1) they are regulated/recruited by Rabs; 2) they are peripheral membrane proteins; 3) they are composed of long rope-like proteins or large oligomeric complexes; 4) they form membrane-bridging complexes; and 5) their initial action precedes and is independent of SNARE function (5–8). Trans-SNARE complex assembly is believed by many in the field to represent the most distal trigger of membrane fusion (9). The current view is that these 4-helix bundles draw the two membranes into very close proximity until ultimately overcoming the hydrostatic barrier to membrane fusion. Sec1/Munc18 proteins are the best candidates for later stage conformational regulators of SNARE complex formation or organization (10, 11).

A functional link between tethers and SNAREs has emerged, and a number of physical interactions between these sets of proteins have been reported (1, 8, 12–18). Current models propose that tethering proceeds upstream of and independently of SNAREs, and then tether–SNARE interactions recruit SNAREs to the site of future membrane fusion and activate or facilitate trans-SNARE interactions. For example, in vitro SNARE assembly studies involving the Rab1 effector p115 and ER/Golgi SNAREs suggested that during Golgi reassembly p115 first mediated membrane tethering through its interactions with other Golgi tethers, and then facilitated trans-SNARE complex formation via direct p115–SNARE interactions (8).

Transport from the ER to the Golgi is one of the most studied steps in the endomembrane system because of its rate-limiting role in protein secretion and quality control, and much of the docking and fusion machinery required for this process have been identified. Rab1 appears to represent a coordinating factor for transport and has a number of effectors (1). These include the putative tethering protein p115 (Uso1p in yeast), which is required for anterograde transport (19). The peripheral membrane protein GM130, another Rab1 effector, and integral-membrane protein D-galactopyranoside; LPC, lysophosphatidylcholine; ER, endoplasmic reticulum.

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4 The abbreviations used are: SNARE, SNAP receptor; SNAP, soluble NSF attachment protein; NSF, N-ethylmaleimide-sensitive factor; VTC, vesicular tubular cluster; COPII, coat complex II; TRAPP, transport protein particle; VSV-G, radiolabeled vesicular stomatitis virus glycoprotein; VSV-G-myc, Myc-tagged nonradioactive vesicular stomatitis virus glycoprotein; NRK, normal rat kidney; TTLC, tetanus toxin light chain; GDI guanine nucleotide dissociation inhibitor; GM130, Golgi matrix protein of 130 kDa; COPI, coatomer; β-COP, coatomer β subunit; HOPS, homotypic fusion and vacuole protein sorting; BSA, bovine serum albumin; IPTG, isopropyl 1-thio-β-β-d-galactopyranoside; LPC, lysophosphatidylcholine; ER, endoplasmic reticulum.
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Golgi vesicle protein Giantin are proposed to work in complex with p115 as tethers and are required for anterograde ER-to-Golgi transport at a late step as well as intra-Golgi transport (19–21). The seven-member TRAPP complex includes subunits mBet3 and Sedlin, is required for anterograde transport, and may represent a second tethering system for ER-to-Golgi transport and a guanine nucleotide exchange factor for Rab1 (22–24). The SNARe machinery consists of Syntaxin 5, Membrin, rBet1, and Sec22b (25). These four proteins are required for ER-to-Golgi transport and form a well-characterized quaternary complex that has served as a prototype for intracellular SNARe complexes (26, 27). Syntaxin 5 interacts with the Sec1/Munc18 protein rSly1; both the interaction and rSly1 itself are essential to fusion (11).

Membrane fusion in ER-to-Golgi transport was initially thought to occur between ER-derived COPII vesicles and the cis-Golgi. However, live imaging studies demonstrated that cargo transport in mammalian systems is primarily mediated not by small vesicles but by larger vesicular tubular clusters (VTCs) that travel along microtubules to the Golgi (28, 29). Some VTCs may be long lived and relatively immobile sorting stations that give rise to the transient, mobile structures (30). ER-to-Golgi cargo transport must involve at least two membrane fusions as follows: one to transfer cargo from COPII vesicles into VTCs in the periphery of the cell, and a second to transfer cargo from Golgi-centric VTCs into the cis-Golgi (or alternatively to create the cis-Golgi de novo). This complexity of fusion events has not been dissected using in vitro reconstitutions of ER-to-Golgi transport (1, 31, 32). One simple model of VTC biogenesis holds that COPII vesicles fuse with each other homotypically to form nascent VTCs. Nascent VTCs may then expand by further fusion with COPII vesicles. Consistent with this model, VTCs (33, 34) or their apparent precursors (35) appear very near ER exit sites, and COPII vesicles contain all machinery necessary for homotypic tethering and fusion when incubated in vitro (36). Thus, homotypic COPII vesicle fusion appears to represent at least one pre-Golgi membrane fusion contributing to VTC biogenesis. Little is known about the mechanism of homotypic COPII vesicle fusion other than that it requires the Syntaxin 5 SNARe complex, rSly1, and the TRAPP subunit mBet3 (36, 37). Whether p115, GM130, or other Rab effectors are involved in homotypic COPII vesicle fusion has not been determined.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Affinity-purified rabbit anti-Syntaxin 5 and rBet1 antibodies (25) and anti-p115 (19) were described previously. Antibodies used in functional assays were purified on antigen affinity columns in the absence of detergent and dialyzed extensively in 25/125 Buffer (25 mM HEPES, pH 7.2, 125 mM potassium acetate). Antibody concentration was determined by absorbance at 280 nm, assuming 1 OD unit equals 0.8 mg/ml of rabbit IgG. Anti-Myc antibody was purified on protein A–Sepharose from tissue culture medium employed to grow the 9E10 hybridoma and then biotinylated with sulfosuccinimidyl Biotin (Pierce). Anti-Syntaxin 5 and preimmune Fabs were produced by papain digestion using a kit from Pierce. Following near-complete IgG digestion, intact IgG and Fc fragments were removed by passing the reaction over a protein A column. Gel analysis confirmed that our Fab preparations lacked any detectable antibody heavy chain (not shown). Anti-GM130 was a kind gift from Dr. Martin Lowe (University of Manchester). Anti-p24, Sec23, and β-COP were produced in rabbits against synthetic peptides, and then affinity-purified using columns with the immobilized peptides (p24 peptide, CQRMHKSFFEAKKLV; Sec23 peptide, CQNEERDGVRFSWNWVPS; β-COP peptide, CKKEAGELKPEEITVGPVQK). Anti-glyceraldehyde-3-phosphate dehydrogenase was from Santa Cruz Biotechnology (product number sc20357).

Lysophosphatidylcholine (1-lauroyl-2-hydroxy-sn-glycero-3-phosphocholine) was purchased from Avanti Polar Lipids (Alabaster, AL) in powder form, weighed freshly for each use, and dispersed by vortexing for 1 min in 25 mM HEPES, pH 7.2, 125 mM potassium acetate (25/125 buffer) at a concentration of 2 mM. This suspension was added to tethering/fusion reactions at ~240 μM. Previous users of this reagent reported significant inhibition of biological membrane fusion with 100 μM lipid (38); however, we did not observe inhibition until ~200 μM. We speculate that its potency in membrane fusion may depend upon the physical state of the lipid dispersion.

Preparation of Purified Recombinant Proteins—His-α-SNAP wild type and L294A plasmids were obtained from Dr. Phyllis Hansson (Washington University, St. Louis) and expressed in Escherichia coli using pET-28. 3 liters of culture was grown in LB containing 50 μg/ml kanamycin to an absorbance at 600 nm of 0.4–0.6, at which time the cultures were induced with 0.5 mM IPTG at 37 °C for 2 h. Induced bacterial pellets were resuspended in 60 ml of 20 mM Tris, pH 7.4, 250 mM NaCl, 5% glycerol, and protease inhibitors (Complete EDTA-free; Roche Applied Science), disrupted twice with a French press, and clarified by centrifugation at 20,000 × g for 20 min followed by 100,000 × g for 1 h. The final lysates were applied to a 2.5 ml nickel-nitrioltriacetic acid–agarose column (Qiagen) equilibrated in 50 mM Tris, pH 7.6, 0.3 M NaCl, washed, and eluted with the same buffer containing 25 mM imidazole and then with a 50-ml gradient from 25 to 250 mM imidazole. Essentially homogeneous α-SNAP protein eluted at around 100 mM imidazole, and was pooled, dialyzed extensively against 25/125 Buffer, and stored in aliquots at ~80 °C until use. Quantitation of all recombinant proteins was performed by Coomassie staining in SDS gels relative to BSA standards.

Vector pET3 expressing his-Rab-GDI was obtained from Dr. William Balch (Scripps Institute, La Jolla, CA). 3 liters of cultured E. coli strain BL21 harboring this construct was grown at 37 °C in LB containing 100 μg/ml ampicillin to an absorbance at 600 nm, at which time the culture was shifted to 15 °C for 30 min and then induced with 1 mM IPTG for 2 h. Induced bacterial pellets were resuspended in 60 ml of French Press Buffer (50 mM Tris, pH 8.0, 0.1 M NaCl, 1 mM EDTA and protease inhibitors as above), French pressed twice, and clarified by centrifugation at 20,000 × g for 20 min followed by 100,000 × g for 1 h. The final lysate was applied to a 2.5-ml nickel-nitrioltriacetic acid–agarose column equilibrated in 50 mM Tris, pH 7.6, 0.3 M NaCl, washed, and eluted with the same buffer containing 25 mM imidazole and then with a 50-ml gradient from 25 to 250
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mm imidazole. Rab-GDI eluted in the 25 mm imidazole buffer and was pooled, dialyzed extensively against 25/125 Buffer, and stored in aliquots at −80 °C until use. Tetanus toxin light chain (TTLC) was expressed as a control protein from vector pQE-3 by induction with 0.5 mM IPTG for 2 h at 37 °C in LB containing 100 µg/ml ampicillin and 50 µg/ml kanamycin. The induced bacterial pellet was resuspended and processed similarly to that of Rab-GDI. Like Rab-GDI, TTLC eluted in the 25 mM imidazole eluate and gave a similar yield of protein per liter of culture as the Rab-GDI construct.

GST-Sar1a T39N in a pGEX vector was obtained from Drs. Jinoh Kim and Randy Schekman (University of California, Berkeley). 3 liters of culture was grown in LB containing 100 µg/ml ampicillin at 37 °C to an optical density of 0.4–0.6, and then protein expression was induced by addition of 1 mM IPTG for 2 h. Bacterial pellets were resuspended in French Press Buffer containing 1 mM DTT, 10 mM magnesium acetate, and stored at −70 °C.

Co-isolation and Heterotrimerization Assays—Experiments were carried out as described before (36). Briefly, the VSV-G-myc-expressing cells and pulse-radiolabeled VSV-G* containing cells were permeabilized by scraping with a rubber policeman, then washed, and resuspended at 125 µl/10-cm plate in 50/90 Buffer (50 mM Hepes, pH 7.2, 90 mM potassium acetate). A first-stage (vesicle release) incubation to produce enough vesicles for an 18-point assay contained 1.45 ml and consisted of 320 µl of water, 50 µl of 0.1 M magnesium acetate, 100 µl of ATP-regenerating system, 30 µl of ATP-regenerating system, 9 µl of 1 M Hepes, pH 7.2, 60 µl of Calcium Buffer, 150 µl of dialyzed rat liver cytosol, 165 µl of 25/125 Buffer or test proteins dialyzed in this buffer, and 75 µl of salt-extracted VSV-G-myc-expressing cells. Vesicle release, tethering, and fusion were allowed to occur in a single 90-min incubation at 32 °C because we wanted the test proteins to be present from the inception of vesicle biogenesis. After vesicle release/tethering/fusion, cells were removed by centrifugation at 4,000 × g as above. The vesicle-containing supernatant was supplemented with 5 µg of anti-Myc antibody and incubated for 2 h with agitation. The suspension was then cleared by centrifugation at 4,000 × g for 1 min followed by addition of 15 µl (packed) of protein A-Sepharose beads (pre-blocked in 5 mg/ml milk and 5 mg/ml PVP-40T in 25/125 Buffer) and further mixing at 4 °C for 4–6 h or overnight. Beads were washed four times by centrifugation at 375 × g for 1 min and resuspension in 25/125 Buffer containing 1 mg/ml BSA. During the last wash, resuspended beads were transferred to a fresh tube. After the washes, proteins were eluted from the beads by addition of 50 µl of 0.1 M glycine, pH 2.5, mixing, centrifugation at 375 × g for 1 min, and removing the supernatant to a tube containing 7.5 µl of 2 M Tris, pH 8.0. Elution was repeated twice more into this eluate pool (157.5 µl of total eluate) and the eluate pool was placed into a Microcon YM-10 ultrafiltration device (Amicon) and centrifuged to dryness. The sample on the membrane was dissolved in 25 µl of SDS sample buffer and analyzed by 4–20% gradient SDS-polyacrylamide gels and immunoblotting.

RESULTS

A preceding report described assays to detect homotypic COPII vesicle tethering and fusion (36). Briefly, two COPII vesicle populations, marked in different ways, are generated from permeabilized normal rat kidney (NRK) cells. One population of vesicles contains Myc-tagged VSV-G ts045 (VSV-G-myc), and the second population of vesicles contains untagged but radiolabeled VSV-G* (see Fig. 1A). After the vesicles are released in a first-stage incubation, the permeabilized donor cells are removed, and the differently marked vesicle release using biotinylated anti-Myc antibody and streptavidin-Sepharose, and co-precipitated VSV-G* was quantified.

Immunoblotting of Transport Intermediates—Only VSV-G-myc-expressing cells were employed because radioactivity was inconsequential to this assay. After scraping and washing, the cells were resuspended in 0.5 ml of 50/90 Buffer per 10-cm plate. This suspension was diluted with 0.5 ml of 50/90 Buffer containing 1.5 M KCl and 1 mg/ml BSA, and the suspension was kept on ice and resuspended once every 2–3 min for 15 min. The suspension was next diluted with 1 ml of 50/90 containing 1 mg/ml BSA, and the cells were sedimented by centrifugation at 4,000 × g for 3 min. Cell pellets were then resuspended in 2 ml of 50/90 Buffer containing 1 mg/ml BSA, sedimented once more, and finally resuspended in 100 µl of 50/90 Buffer per original plate. Cells treated in this way were functional for vesicle release, co-isolation, and heterotrimer assays (36). Each immunoisolation condition involved a reaction volume of 600 µl and contained the following: 96 µl of water, 15 µl of 0.1 M magnesium acetate, 30 µl of ATP-regenerating system, 9 µl of 1 M Hepes, pH 7.2, 60 µl of Calcium Buffer, 150 µl of dialyzed rat liver cytosol, 165 µl of 25/125 Buffer or test proteins dialyzed in this buffer, and 75 µl of salt-extracted VSV-G-myc-expressing cells. Vesicle release, tethering, and fusion were allowed to occur in a single 90-min incubation at 32 °C because we wanted the test proteins to be present from the inception of vesicle biogenesis. After vesicle release/tethering/fusion, cells were removed by centrifugation as above. The vesicle-containing supernatant was supplemented with 5 µg of anti-Myc antibody and incubated for 2 h with agitation. The suspension was then cleared by centrifugation at 4,000 × g for 1 min followed by addition of 15 µl (packed) of protein A-Sepharose beads (pre-blocked in 5 mg/ml milk and 5 mg/ml PVP-40T in 25/125 Buffer) and further mixing at 4 °C for 4–6 h or overnight. Beads were washed four times by centrifugation at 375 × g for 1 min and resuspension in 25/125 Buffer containing 1 mg/ml BSA. During the last wash, resuspended beads were transferred to a fresh tube. After the washes, proteins were eluted from the beads by addition of 50 µl of 0.1 M glycine, pH 2.5, mixing, centrifugation at 375 × g for 1 min, and removing the supernatant to a tube containing 7.5 µl of 2 M Tris, pH 8.0. Elution was repeated twice more into this eluate pool (157.5 µl of total eluate) and the eluate pool was placed into a Microcon YM-10 ultrafiltration device (Amicon) and centrifuged to dryness. The sample on the membrane was dissolved in 25 µl of SDS sample buffer and analyzed by 4–20% gradient SDS-polyacrylamide gels and immunoblotting.
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FIGURE 1. Anti-Syntaxin 5 antibodies inhibit homotypic tethering. A, a schematic of co-isolation and heterotrimer assays for homotypic COPII vesicle tethering and fusion. See text for description. B, anti-Syntaxin 5 IgG selectively inhibits the heterotrimerization assay at a low concentration and both co-isolation and heterotrimerization at a higher concentration. Two COPII vesicle populations were produced from permeabilized NRK cells during a first-stage incubation as described before (36). The two vesicle populations were then combined and preincubated for 20 min on ice in the presence or absence of the indicated concentrations of affinity-purified anti-Syntaxin 5 antibody or nonimmune IgG. After the preincubation, one sample was left on ice, and the others were subjected to a second-stage incubation at 32 °C for 60 min to allow tethering and fusion. After the second-stage incubation, samples were either subjected to anti-Myc immunoprecipitation of intact vesicles (co-isolation, gray bars) or anti-myc immunoprecipitation of detergent-solubilized VSV-G trimers (heterotrimers, black bars). After quantitation of recovered VSV-G by each sample by SDS-PAGE and phosphorimaging, the arbitrary radioactivity counts for each assay process were normalized to the 100% value obtained in the reaction incubated at 32 °C without antibody addition. C, anti-Syntaxin 5 Fab fragments inhibit both co-isolation and heterotrimerization assays. Methods are similar to B, except that purified Fab fragments were utilized instead of IgG, and the negative control consisted of a fully incubated reaction where an equal number of nontransfected NRK cells were substituted for VSV-G-myc-transfected cells. D, anti-Syntaxin 5 Fabss do not significantly affect the immunosolubilization efficiency of VSV-G-myc vesicles or solubilized VSV-G-myc-containing trimers. An experiment similar to that in C was carried out except that the no bio negative control represents reactions that were fully incubated and contained both VSV-G-myc and VSV-G* vesicle populations, but were immunosolubilized/precipitated using nonbiotinylated anti-Myc antibody (and thus could not specifically bind streptavidin-Sepharose). Treated samples received 350 nM anti-Syntaxin 5 Fab. After SDS-PAGE as usual, immunoprecipitates were transferred to nitrocellulose for autoradiography, followed by immunoblotting with anti-Myc antibody, and the recovery of VSV-G-myc in each sample was quantitated by chemiluminescence. The graph shows VSV-G* recoveries (co-isolation and heterotrimer assay autoradiography results) as gray and black bars as usual, but with the VSV-G-myc recoveries indicated by white bars set behind each of the gray and black bars. B, the results from three separate experiments with similar results were combined to make the figure. C, a single representative experiment is shown; similar results were obtained on two occasions. D is from a single experiment. In these and subsequent experiments, histograms represent means of 2–6 determinations ± S.E., a single asterisk denotes experimental effects where p < 0.1 when compared with positive control (pos) in a two-tailed t test assuming equal variances, and a double asterisk represents effects where p < 0.05.

 Vesicle populations are combined and incubated together in a second-stage incubation during which tethering and fusion between the two vesicle populations occurs. These events can be detected subsequently as follows: VSV-G*–containing radiolabeled vesicles that interact with VSV-G-myc vesicles can be co-immunoprecipitated, intact, from the supernatant with anti-Myc antibody and detected with autoradiography. As depicted in Fig. 1A, this “co-isolation” assay scores tethering or a mixture of tethering and fusion but cannot distinguish these possibilities. On the other hand, heterotrimeric VSV-G molecules that contain both VSV-G-myc and VSV-G* form only following fusion and membrane mixing and can be detected specifically by immunoprecipitation in the presence of detergent. This so-called “heterotrimer” assay specifically reports fusion; by utilizing both assays, the tethering and fusion processes can be distinguished and dissected. Extensive analyses employing these assays indicated that other types of vesicles such as Golgi, ER, pre-existing VTCs, and COPI vesicles were either not present or not required for in vitro generation of nascent VTCs, providing the most direct evidence to date that COPII vesicles can tether and fuse homotypically to generate pre-Golgi intermediates de novo (36).

A Role for SNAREs Upstream of Fusion in Homotypic COPII Vesicle Tethering—Fusion of COPII vesicles to form pre-Golgi intermediates requires the ER-Golgi SNARE complex, because the heterotrimer assay was inhibited by anti-Syntaxin 5 and anti-rSly antibodies and by addition of ~1 μM of dominant-negative α-SNAP L294A (36). The inhibitory properties of α-SNAP L294A arise from its association with SNARE complexes and NSF but failure to stimulate NSF ATPase activity, creating a block in SNARE recycling (39). Curiously, α-SNAP L294A significantly inhibited the co-isolation assay as well as the heterotrimer assay (36). Although we speculated that this might be indicative of an unexpected role for SNAREs upstream of fusion, we could not rule out the simpler possibility that fusion was required for a robust co-isolation signal.
To investigate the potential role for SNAREs upstream of fusion more thoroughly, we tested anti-Syntaxin 5 antibodies at different concentrations and monitored both the co-isolation and heterotrimer signals. As shown in Fig. 1B, 100 nM anti-Syntaxin 5 antibody fully inhibited the heterotrimer assay to near background without any inhibition (or perhaps even a slight stimulation) of the co-isolation signal performed in parallel. This demonstrates that membrane fusion is not a requirement for a robust co-isolation signal. On the other hand, 175 nM of the antibody caused an ~40% inhibition (~66% relative to the ice background) of co-isolation (Fig. 1B). Thus, it appears that anti-Syntaxin 5 antibody interferes with an earlier and distinct Syntaxin 5-dependent process when employed at higher concentrations. The effects on co-isolation are immune-specific because protein A-purified nonimmune IgG (Fig. 1B) or other irrelevant antibodies (data not shown) can be included with no inhibition of co-isolation. We do not know why the antibody inhibited co-isolation less potently than the fusion assay, but we speculate that perhaps lower affinity epitopes mediate that inhibition.

To confirm that the inhibition of co-isolation was not an indirect steric consequence of antibody binding, we produced monovalent Fab fragments from the affinity-purified bivalent antibodies tested in Fig. 1B. As shown in Fig. 1C, anti-Syntaxin 5 Fab fragments inhibited both the co-isolation and heterotrimer assays by about 40% when employed at limiting concentrations (227 nM). At high concentrations (625 nM), co-isolation was inhibited by about 70%, and heterotrimerization was inhibited to background. As expected, nonimmune Fabs had no effect. The increased relative potency of the anti-Syntaxin 5 Fab in the co-isolation assay (Fig. 1C), compared with that of whole IgG (Fig. 1B), could be due to better access of the smaller reagent to the relevant epitopes; alternatively, it could be due to elimination of the slight stimulatory effect caused by the intact IgG. Most importantly, the inhibition of both co-isolation and heterotrimer assays by anti-Syntaxin 5 Fabs confirms that Syntaxin 5 functions both in fusion, as expected, as well as upstream of fusion in a vesicle docking/tethering process. Fig. 1D demonstrates that the inhibition of VSV-G* co-isolation and heterotrimerization by anti-Syntaxin 5 Fabs was not attributable to a decreased yield of immunoisolated VSV-G-myc vesicles or trimers.

To confirm further that the two assays measure different processes and that inhibition of fusion does not automatically or indirectly inhibit the co-isolation assay, we employed lysophosphatidylcholine (LPC), an inverted cone-shaped lipid known to inhibit biological membrane fusion by disfavoring negative membrane curvature and lipid stalk formation (38). As shown in Fig. 2A, LPC added as an aqueous dispersion prior to the tethering/fusion incubation inhibited the heterotrimer signal by up to 80% and was accompanied by a minor stimulatory effect on co-isolation. The minor effect on co-isolation appeared to be accounted for by a comparable increase in the yield of immunoisolated VSV-G-myc vesicles (not shown). The substantial inhibition of the heterotrimer assay without inhibition of co-isolation further established that the relative selectivity of inhibitors between the two assays may be employed to distinguish functional roles in fusion from upstream roles in vesicle tethering. As a consequence, the ~40% inhibition of both assays by 227 nM anti-Syntaxin 5 Fab (Fig. 1C) indicates that this reagent acts upstream of fusion.

We next re-examined the previously observed effects of α-SNAP L294A on co-isolation. As shown in Fig. 2B, inclusion of the mutant L294A at 1 μM inhibited the heterotrimer assay very significantly with only a slight perturbation of co-isolation. Thus, at this concentration, α-SNAP L294A is a selective fusion inhibitor, as would be expected of a reagent that reduces the availability of SNAREs for trans-SNARE complex formation. However, 3 μM L294A significantly inhibited the co-isolation assay as well, again implying a SNARE dependence of the homotypic tethering process. The inhibition of co-isolation, although less potent, was nonetheless specific, because wild type α-SNAP at the same concentration did not inhibit co-
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isolation (Fig. 2B, white bar). The higher concentration of α-SNAP L294A required to inhibit co-isolation may reflect a need for a more complete block of SNARE recycling. Alternatively, α-SNAP L294A could inhibit tethering by a distinct mechanism, for example by direct competition with tethering factors for binding to SNAREs. In either case, the sensitivities of the co-isolation and heterotrimer assays to SNARE reagents indicate that SNARE-dependent processes are required for both tethering and fusion.

SNARE Status Dictates Tether Recruitment and/or Retention on COP II Vesicles—One potential role for SNAREs upstream of fusion would be to provide a “signal” for tether recruitment or retention at fusogenic membrane sites. If disrupted tether-SNARE interactions were responsible for the inhibition of tethering by α-SNAP L294A, then we might expect to see a decreased content of tether molecules on L294A-treated vesicles. To render ongoing tether–SNARE interactions as rate-limiting as possible for tether recruitment/retention, we salt-extracted the permeabilized cells prior to vesicle generation. This appeared to render the recruitment or retention of active tethers to focal sites biochemically “visible” on vesicles that could otherwise contain functionally irrelevant pools of these molecules. As shown in Fig. 3A, 0.75 M KCl pretreatment of the cells removed the vast majority of peripheral membrane proteins p115, Sec23, and β-COP from permeabilized cells. The remaining minority of protein must have been membrane-bound, and not just trapped, because glyceraldehyde-3-phosphate dehydrogenase, a cytosolic protein, was completely removed. GM130 was incompletely extracted by the salt treatment, perhaps because of its known participation in an insoluble Golgi matrix. Although not apparent, GM130 on pre-Golgi membranes may have been extracted. Integral membrane proteins rBet1 and p24 demonstrate equivalent loading of the relevant membrane compartments. Similar salt-extracted permeabilized cells were demonstrated to be functional for vesicle release, co-isolation, and heterotrimer assays (36).

We next produced COP II vesicles and nascent VTCs from salt-extracted permeabilized cells in the presence or absence of α-SNAP reagents, followed by immunoblotting of the immunosolated transport intermediates. As shown in Fig. 3B, transport intermediates contained the peripheral membrane proteins Sec23, p115, and GM130, and the integral membrane protein VSV-G-myc. The vesicles were produced in a Sar1-dependent manner and were dependent upon VSV-G-myc for specific immuno-isolation. Strikingly, when vesicles were produced in the presence of α-SNAP L294A, the putative tether molecules GM130 and p115 were conspicuously absent. The lack of these Rab1 effectors was not a nonspecific effect of L294A coating membranes and preventing access of peripheral membrane proteins, because 1) vesicles as marked by VSV-G-myc were still generated and 2) the vesicles contained a proportionally normal content of the peripheral membrane protein Sec23. The block in tether recruitment was specific for α-SNAP L294A, because addition of wild type α-SNAP did not affect GM130 recruitment (Fig. 3C). Previous work had indicated that p115 played a role in “programming” COP II vesicles, at the moment of budding, for their ultimate transport to the Golgi (1). The present results are consistent with a programming role because our assay does not distinguish at what point p115 is first recruited in a SNARE-dependent fashion; it could be prior to budding or after, and either would result in transport intermediates lacking p115. However, because addition of α-SNAP L294A after vesicle formation blocks tethering (Fig. 2B), we can conclude that tethering remains dependent upon tether–SNARE interactions beyond the budding or programming stage.

Although it is established that p115 is recruited to budding COP II vesicles (1), we were somewhat surprised to observe GM130 on our immunosolated COP II vesicles/nascent VTCs, because this protein has been implicated in early Golgi/late VTC events (40) but was not observed on COP II intermediates (20). We wondered whether this Rab1 effector was directly recruited to COP II vesicles from the cytosolic pool (see Fig. 3A, 1st lane) or, on the other hand, was recruited via fusion of
COPII intermediates with Golgi-derived vesicles. Our previous work demonstrated that nascent VTCs acquired Giantin via a COPI-dependent pathway, presumably via heterotypic fusion with Golgi-derived COPII vesicles. When COPI function was blocked by employing β-COP-depleted cytosol, homotypic tethering and fusion still occurred, but the VSV-G-containing fusion product lacked Giantin (36). In contrast, Fig. 3D demonstrates that vesicles produced using the same β-COP-depleted cytosol still contained abundant GM130. This result shows that GM130 is likely recruited to COPII vesicles directly from a cytosolic pool. Together, the results of Fig. 3 demonstrate that recruitment or retention of the Rab1 effectors p115 and GM130 on COPII intermediates is sensitive to the status of SNAREs.

**Rab Effectors Including p115 Are Required for Homotypic COPII Vesicle Tethering**—We have documented that SNARE reagents can inhibit the tethering event upstream of fusion (Figs. 1 and 2) and that this inhibition correlates with a loss of p115 and GM130 recruitment/retention on COPII-dependent transport intermediates (Fig. 3). Thus, it seemed possible that the lack of p115 and/or GM130 was the proximal cause of the failure of tethering in the presence of α-SNAP L294A. However, it is not known whether homotypic COPII vesicle tethering requires p115 and/or GM130. Although Rab1 and p115 were implicated at the inception of a COPII vesicle for programming it for fusion with the Golgi (1), whether Rab effectors have a role downstream of budding in homotypic tethering is unknown. To begin to address this question, we used Rab-GDI to inhibit the association of Rabs with vesicles during the homotypic tethering event. The Rab-GDI was added after vesicle biogenesis at the outset of the second-stage tethering/fusion incubation. As shown in Fig. 4A, recombinant his-Rab-GDI was largely a single ~52-kDa band except for a minor contaminant of slightly larger size. This contaminant was also visible in control His-tetanus toxin light chain, purified by the same protocol. Fig. 4B shows that addition of 0.66 μM of the recombinant proteins had very different effects on COPII vesicle co-isolation. Although the tetanus toxin light chain had no effect, Rab-GDI inhibited the process to near the ice background. This experiment demonstrates that Rab effectors are required after vesicle budding, during the tethering process.

We also tested directly whether the Rab1 effector p115 was required during the homotypic COPII vesicle tethering event. As shown in Fig. 4C, addition of as little as 28 nM of anti-p115 antibody inhibited the co-isolation assay to background levels, making anti-p115 the most potent inhibitor of co-isolation yet discovered. Pre-heating the antibody at 95 °C for 3 min neutralized the inhibitory effect, demonstrating specificity. Because GM130 has been implicated later in the secretory pathway, we did not specifically analyze its potential role in the homotypic tethering, although a dual role with p115 cannot be eliminated. In summary, the results of Fig. 4 indicate that Rab effectors, including p115, play an active post-budding role in homotypic COPII vesicle tethering. Thus, the lack of p115 recruitment or retention to transport intermediates can explain the inhibition of tethering by α-SNAP L294A.

Altogether, our results identify an unanticipated role for SNAREs in tether recruitment, retention, and function on COPII vesicles. Fig. 5 shows two models of how this new role for SNAREs may be integrated into the tethering/fusion cycle. Previous models of tether–SNARE interactions presumed that they functioned downstream of and independently of the tethering event. Although this may also be the case, our work expands the known interdependence of SNAREs and tethers to an earlier step.

**DISCUSSION**

Our data demonstrate that SNAREs play an unanticipated role upstream of fusion in recruiting GM130 and p115 to COPII vesicles for function in homotypic tethering. Furthermore, we demonstrate that Rab effectors, including p115, are required for homotypic COPII vesicle tethering. Thus, the recruitment or retention of p115 on COPII vesicles to allow productive tethering is at least one important functional role of SNAREs upstream of their well known role in fusion. These data lead to
a re-consideration of the relationship between SNAREs and tethers and indicate a higher degree of interdependence of these proteins than previously imagined (see our model in Fig. 5). Unlike in previous models, SNAREs participate actively throughout the tethering and fusion process. One important consequence of this revision is that tethering would only take place at focal sites where fusogenic SNAREs are already present. This would help ensure that nonproductive tethering does not take place. If, on the other hand tethering does occur, the continuing dependence upon SNAREs for tether retention/activation would allow the tether to disengage when SNARE status changes, either because of productive trans-SNARE assembly or because the SNAREs become nonproductively otherwise engaged. This would provide for a potential tether release mechanism that would eliminate “dead end” tethering when fusion fails. Recently another report concluded that SNARE status influences the on-off dynamics of Golgi-localized p115 measured by fluorescence recovery after photobleaching (41). This study provides important confirmation of this principle in vivo; however, it differs from our study because it addresses the bulk dynamics of p115 on the Golgi and is limited because it does not address whether the SNARE-responsive pool of p115 is functionally important either to retain the tether at the proper site or to keep it active for tethering.

p115 is a Rab1 effector with multiple proposed functions and interaction partners. Our work adds to this by demonstrating that p115 is required at a post-budding but pre-tethering stage of homotypic COPII vesicle docking and fusion. This role appears to depend on SNAREs, and because direct p115-SNARE interactions have been established (8), it follows that these p115-SNARE interactions are required for tether recruitment. This is a different role from what was proposed, which assumed that p115-SNARE interactions were important for SNAREpin assembly. However, they are not incompatible. The p115-SNARE interactions could first regulate tether recruitment/retention and activation and then subsequently regulate SNAREpin assembly (see Fig. 5). If both roles are correct, then p115 and SNAREs are seamlessly integrated throughout both tethering and fusion.

GM130 is a Rab1 effector whose presence or function has not been reported previously on COPII intermediates (20) and has been suggested to interact only indirectly with SNAREs (8). Curiously, however, a Chinese hamster ovary cell mutant that is temperature-sensitive for growth and lacks detectable GM130 expression is completely rescued by overexpression of NSF.
(43). Why would NSF serve as an extragenic suppressor for defective or unstable GM130? Our results provide a potentially compelling explanation in that recruitment/retention of GM130 at the membrane may require uncomplexed SNAREs or a SNARE subcomplex that increases in abundance when NSF activity is increased. Overexpression of NSF would create a condition where abundant tether-reactive SNAREs result in more GM130 retention/recruitment to membranes. This may stabilize the GM130 and allow it to escape degradation in the mutant cell line. At this time we do not know whether GM130 directly recognizes the SNARE signal itself or is recruited via interaction with p115, a GM130 binding partner.

We recently determined that mBet3, a component of the mammalian TRAPP complex, was required for homotypic COPII vesicle tethering (37). Thus, two specific putative tether proteins, mBet3 and p115, have now been demonstrated to be required for this process. It is not known if one, both, or neither of these proteins is part of the physical link between tethered COPII vesicles. Tethering may be a multistep process involving multiple regulatory events. More extensive analysis will be required to determine the nature of the roles that both p115 and TRAPP play in the series of events that allow COPII vesicles to recognize and physically link to each other.

Is the role for SNAREs upstream of fusion in tether recruitment, retention, and/or activation conserved at other transport steps involving different SNAREs and tethers? It seems likely. In the initial characterization of α-SNAP L294A, it was noted that this protein seemed to inhibit docking of chromaffin granules when added at higher concentrations than was required for inhibition of exocytosis (39). However, this observation was not pursued because a role for SNAREs in tethering was not expected. Likewise, a recently developed morphological assay for homotypic early endosome docking and fusion found that ~10 μM of α-SNAP L294A was required to inhibit docking/fusion (44). Because that assay did not allow for detection of a tethered but unfused intermediate, the high concentrations required for inhibition may have reflected an inhibition of tethering. There is also ample precedent in the literature for pre-tethering complexes involving both tethers and SNAREs. For example, in yeast vacuole fusion, the SNARE Vam7p partially stabilizes the HOPS tethering complex on the vacuole prior to tethering (45), and the tethering and fusion functions may be integrated into a single HOPS-SNARE complex (42, 46). It is possible that some kind of SNARE signal is an integral component in the tethering of many types of vesicles prior to SNARE-dependent fusion.

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