Requirement for Golgi-localized PI(4)P in fusion of COPII vesicles with Golgi compartments

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ABSTRACT The role of specific membrane lipids in transport between endoplasmic reticulum (ER) and Golgi compartments is poorly understood. Using cell-free assays that measure stages in ER-to-Golgi transport, we screened a variety of enzyme inhibitors, lipid-modifying enzymes, and lipid ligands to investigate requirements in yeast. The pleckstrin homology (PH) domain of human Fapp1, which binds phosphatidylinositol-4-phosphate (PI(4)P) specifically, was a strong and specific inhibitor of anterograde transport. Analysis of wild type and mutant PH domain proteins in addition to recombinant versions of the Sac1p phosphoinositide-phosphatase indicated that PI(4)P was required on Golgi membranes for fusion with coat protein complex II (COPII) vesicles. PI(4)P inhibition did not prevent vesicle tethering but significantly reduced formation of soluble n-ethylmaleimide sensitive factor adaptor protein receptor (SNARE) complexes between vesicle and Golgi SNARE proteins. Moreover, semi-intact cell membranes containing elevated levels of the ER-Golgi SNARE proteins and Sly1p were less sensitive to PI(4)P inhibitors. Finally, in vivo analyses of a *pik1* mutant strain showed that inhibition of PI(4)P synthesis blocked anterograde transport from the ER to early Golgi compartments. Together, the data presented here indicate that PI(4)P is required for the SNARE-dependent fusion stage of COPII vesicles with the Golgi complex.

INTRODUCTION

The secretory pathway is responsible for delivery of proteins and lipids from their site of synthesis at the endoplasmic reticulum (ER) to the cell surface and the many membrane-bound compartments that comprise the endomembrane system. Transport between these compartments is mediated by membrane vesicles and tubules that bud from a donor membrane and selectively target to and fuse with an acceptor membrane. This process is vital for cell growth and for maintenance of intracellular compartments as distinct biochemical environments. Many of the molecular mechanisms that underlie membrane transport are highly conserved between diverse intracellular

trafficking events as well as among eukaryotic species (Bonifacino and Glick, 2004).

After synthesis of secretory molecules at the ER, folded cargo proteins are packaged into coat protein complex II (COPII)-derived transport vesicles (Sato and Nakano, 2007). In yeast, vesicles then traffic toward the Golgi apparatus and tether to *cis*-Golgi membranes in a process that depends on Ypt1p, Uso1p, the Grh1/Bug1p complex, and the transport protein particle (TRAPP)-I complex (Sztul and Lupashin, 2006; Behnia et al., 2007). Vesicle fusion ensues as cognate soluble n-ethylmaleimide sensitive factor adaptor protein receptor (SNARE) proteins assemble into *trans*-SNARE complexes regulated by the function of the SM protein Sly1p (Sudhof and Rothman, 2009) and the SNARE dissociation chaperones Sec17p (α-SNAP) and Sec18p (NSF) (Wickner and Schekman, 2008). Current evidence indicates that the vesicle-SNAREs involved in this process are Bet1p and Bos1p, whereas Sed5p and Sly1p act on the Golgi compartment (Cao and Barlowe, 2000). The R-SNARE Sec22p can function in this fusion reaction from either vesicles or Golgi membranes (Liu and Barlowe, 2002). A complete understanding of the mechanisms underlying ER-to-Golgi transport is limited in part, however, by a lack of knowledge of the role of membrane lipids in COPII vesicle budding, tethering, and fusion with *cis*-Golgi compartments.

In addition to well-recognized structural roles in forming membrane bilayers and establishing subcellular compartments within

eukaryotic cells, specific lipid species are known to regulate membrane dynamics (van Meer *et al.*, 2008). Among these lipids, diacylglycerol (DAG) and the phosphoinositides (phosphorylated derivatives of phosphatidylinositol [PI]) have been implicated in diverse cellular processes, including membrane deformation and vesicle trafficking (Gomez-Fernandez and Corbalan-Garcia, 2007; Strahl and Thorner, 2007; Mulet *et al.*, 2008). Few studies, however, have addressed the role of specific lipid species in transport between ER and Golgi compartments.

In this study we investigated the lipid requirements for ER-to-Golgi transport in the budding yeast *Saccharomyces cerevisiae*, focusing on the role of phosphoinositides. In vitro budding, tethering, and fusion assays were used to screen phosphoinositide binding domains, enzymes that modify phosphoinositides, and inhibitors of enzymes involved in phosphoinositide metabolism. We found that the pleckstrin homology (PH) domain of human Fapp1, which binds specifically to PI(4)P (phosphatidylinositol-4-phosphate), was a potent and specific inhibitor of ER-to-Golgi transport. Stage-specific assays demonstrated that PI(4)P was required at the Golgi compartment for fusion of COPII vesicles with Golgi acceptor membranes. Moreover, reduction of PI(4)P levels significantly decreased formation of SNARE complexes between vesicle and Golgi SNARE proteins. Based on the results presented in this study, we propose that PI(4)P is an important regulator of COPII vesicle fusion with Golgi membranes.

RESULTS

Characterization of inhibitors of ER-to-Golgi transport

To identify specific lipid requirements for ER-to-Golgi trafficking, we screened an array of lipid-binding domains, enzymes, and enzyme inhibitors in cell-free assays to monitor influences on COPII vesicle budding, vesicle tethering, or vesicle fusion with Golgi acceptor membranes. The following lipid-binding domains were tested: the PI(4)P-binding PH domain of human Fapp1 (Dowler *et al.*, 2000; Stahelin *et al.*, 2007); a tandem repeat of the PI(3)P-binding FYVE domain of Eea1 (Gillooly *et al.*, 2000); the PI(4,5)P$_2$-binding ENTH domain of epsin (Rosenthal *et al.*, 1999); the PI(4,5)P$_2$-binding marcks-effector domain (MED) (Wang *et al.*, 2001); and the DAG-binding C1b domain of protein kinase C (Johnson *et al.*, 2000). In addition, we tested a PI-specific phospholipase C (PI-PLC) from *Bacillus cereus* (Ross *et al.*, 1992) that converts PI into DAG and inorganic phosphate; and the PLC inhibitor U73122, which prevents the conversion of PI(4,5)P$_2$ into DAG and IP$_3$ by Plc1p, the only known yeast PLC (Bleasdale *et al.*, 1990; Jun *et al.*, 2004). The concentrations of inhibitors used were in the same range as reported to inhibit other in vitro trafficking reactions (Fratti *et al.*, 2004; Jun *et al.*, 2006).

Inhibitors were initially screened for defects of overall ER-to-Golgi transport. In this assay, washed semi-intact cell membranes containing translocated [^{35}S]glyco-pro-alpha-factor (gpαf) were pretreated with indicated inhibitors for 20 min at 4°C. Reactions were then incubated for 1 h at 23°C with purified transport factors to drive transport of gpαf to the Golgi complex (Barlowe, 1997). Upon delivery to the Golgi complex, gpαf receives outer-chain α1,6-mannose residues that can be immunoprecipitated with anti-1,6-mannose-specific serum to quantify gpαf transport (Baker *et al.*, 1988). As observed in Figure 1A, U73122 and PH strongly inhibited ER-to-Golgi transport, whereas C1b, PI-PLC, and FYVE moderately inhibited the reaction. We next determined the specific stage(s) of ER-to-Golgi transport (budding, tethering, or fusion) that were affected by each inhibitor.

To measure COPII vesicle fusion with Golgi membranes, we performed two-stage fusion reactions. In the first stage, COPII vesicles

containing [^{35}S]gpαf were generated from microsomes and separated from donor ER membranes by centrifugation (Cao and Barlowe, 2000). In the second stage, washed semi-intact cell membranes pretreated with indicated inhibitors were incubated with [^{35}S]gpαf containing vesicles in the presence of fusion factors for 1 h at 23°C (Cao and Barlowe, 2000). As shown in Figure 1B, U73122, PH, and PI-PLC strongly inhibited fusion of COPII vesicles with Golgi acceptor membranes, whereas C1b and FYVE moderately inhibited the reaction. The no acceptor membrane control (no Acpt) reflects the level of Golgi membranes that carry over with COPII vesicles from the microsome budding reaction.

We next determined whether the inhibitors influenced COPII vesicle budding or vesicle tethering to Golgi membranes. For this assay, washed semi-intact cell membranes containing [^{35}S]gpαf were pretreated with indicated inhibitors and then incubated for 30 min at 23°C with COPII proteins to drive vesicle budding (Cao *et al.*, 1998). Because freely diffusible vesicles can be separated from ER and Golgi membranes by centrifugation, budding and Uso1p-dependent tethering of budded vesicles to Golgi membranes can be measured by quantifying the level of [^{35}S]gpαf-containing diffusible vesicles in the supernatant fraction (Cao *et al.*, 1998). As shown in Figure 1C, U73122 was a strong inhibitor of budding, whereas FYVE and PI-PLC were moderate inhibitors of budding. None of the inhibitors produced a strong influence on vesicle tethering to Golgi membranes. We observed, however, that the PH and C1b domains caused an increase in diffusible vesicles. We interpret this increase in diffusible vesicles to be caused primarily by a block in vesicle fusion with Golgi membranes. To ensure that these effects did not arise from a loss in membrane integrity, protease protection of luminal [^{35}S]gpαf was assessed after inhibitor treatments. As shown in Figure 1D, none of the inhibitors compromised the integrity of ER membranes at the concentrations tested, including 80 μM U73122 and 1 nM PI-PLC (unpublished data).

In summary, the tested compounds could be placed into three general classes. The first class contains the PI(4,5)P$_2$ binding domains, ENTH and MED, which did not have a significant effect on ER-to-Golgi transport. The second class comprised the inhibitors U73122, PI-PLC, and FYVE, which targeted both budding and fusion. At higher concentrations, U73122 and PI-PLC were strong inhibitors of budding and fusion, whereas FYVE produced modest inhibition of both of these stages. The last class of inhibitors included the PH and C1b domains that acted specifically at the vesicle fusion stage, with the PH domain acting as a more potent inhibitor. Further study of the inhibitors U73122, PI-PLC, C1b, and FYVE will be necessary to understand their mechanisms of inhibition. We decided to focus on the role of PI(4)P in fusion of COPII vesicles because PI(4)P is prevalent in the early secretory pathway (D'Angelo *et al.*, 2008) and because the PH domain acted potently and specifically at the vesicle fusion stage.

PI(4)P is required at the Golgi complex for SNARE-mediated fusion with COPII vesicles

To determine whether the observed inhibition with the PH domain was specific for PI(4)P, we compared the wild-type PH domain (PH^{WT}) used in Figure 1 to a mutant (PH^{N,K}) that does not bind PI(4)P (Dowler *et al.*, 2000; Blumental-Perry *et al.*, 2006). Titration of these binding domains in one-stage transport reactions showed that PH^{WT} is a dose-dependent transport inhibitor, whereas PH^{N,K} did not inhibit transport even at the highest concentrations tested (Figure 2A). Furthermore, in reactions comparing one-stage transport, vesicle budding, and vesicle tethering (Figure 2B), treatment with 5 μM PH^{N,K} did not have detectable effects on transport,
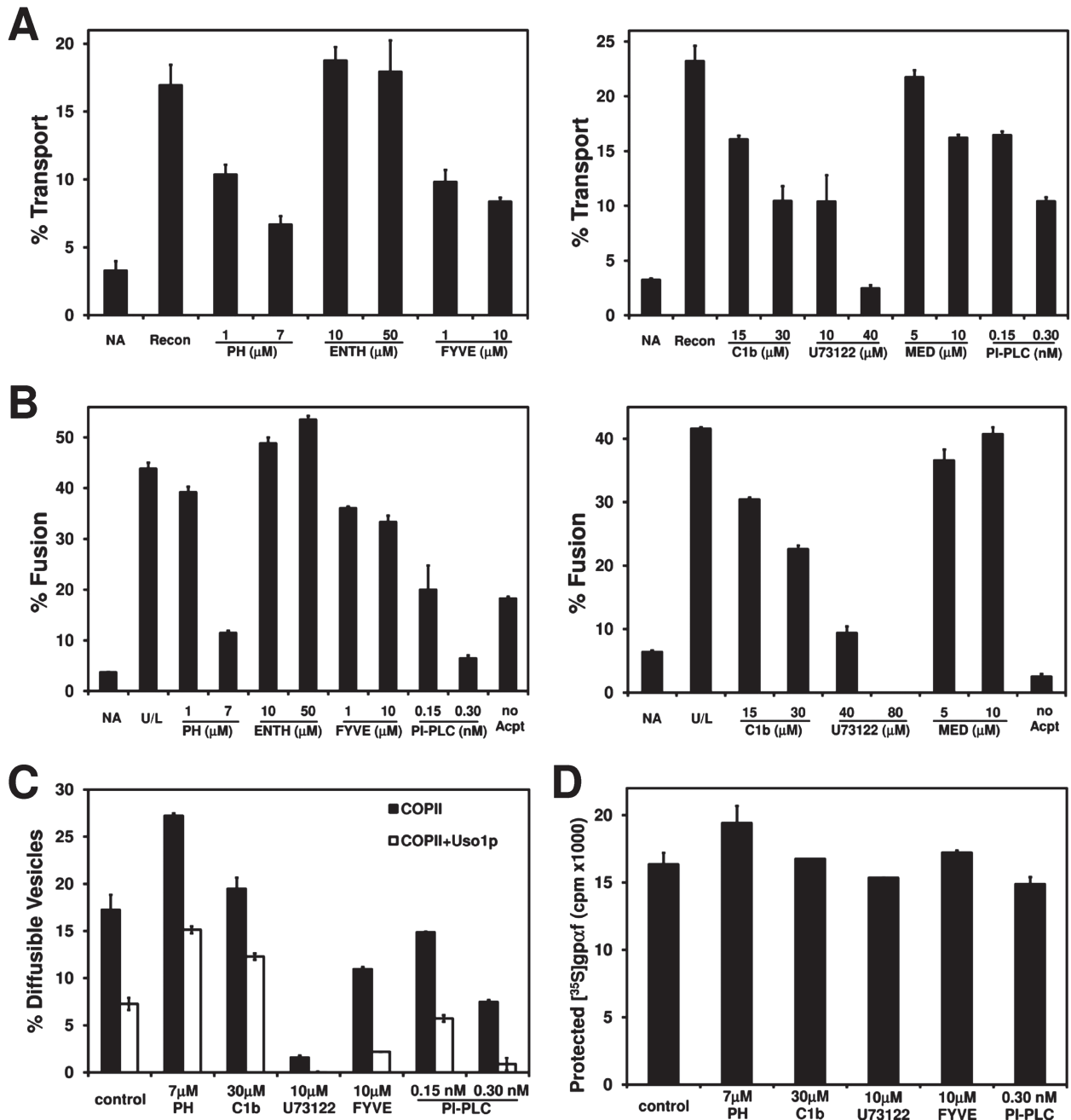
FIGURE 1: Screen for inhibitors of ER-to-Golgi transport. (A) Washed semi-intact cells containing [^{35}S]gpαf were pretreated with indicated inhibitors for 20 min at 4°C. Transport reactions were then incubated with Recon proteins (COPII, Uso1p, and LMA1) and an ATP regeneration system at 23°C for 1 h. The amount of Golgi-modified [^{35}S]gpαf was measured to determine transport efficiency. NA is the background level of transport in the absence of transport factors. (B) Semi-intact cell acceptor membranes were pretreated with indicated inhibitors for 20 min at 4°C and then incubated with COPII vesicles containing [^{35}S]gpαf in the presence of fusion factors (U/L: Uso1p and LMA1) and an ATP regeneration system at 23°C for 1 h. Golgi-modified [^{35}S]gpαf was measured to determine fusion efficiency. The no Acpt condition represents the background level of fusion in the absence of semi-intact cell acceptor membranes. (C) Pretreated, semi-intact cells as in panel A were incubated with COPII or COPII plus Uso1p for 30 min at 23°C. To measure budding (black bars) or tethering (white bars), diffusible vesicles containing [^{35}S]gpαf were separated from semi-intact cell membranes by centrifugation. (D) Mock-budding reactions as in panel C were treated with trypsin, and total protease protected [^{35}S]gpαf was quantified to assess membrane integrity.

whereas 5 μM PH^{WT} inhibited transport and caused an apparent increase in diffusible vesicles (also observed in Figure 1C). Because tethering was largely unaffected (Figure 2B), we conclude that this effect was caused primarily by inhibition of vesicle fusion.

Tethered vesicles could be prevented from fusing with Golgi membranes if formation of *trans*-SNARE complexes was blocked or if assembled *trans*-SNARE complexes could not catalyze membrane fusion. To determine whether PH^{WT} treatment prevents formation of *trans*-SNARE complexes, we used an assay that measures the
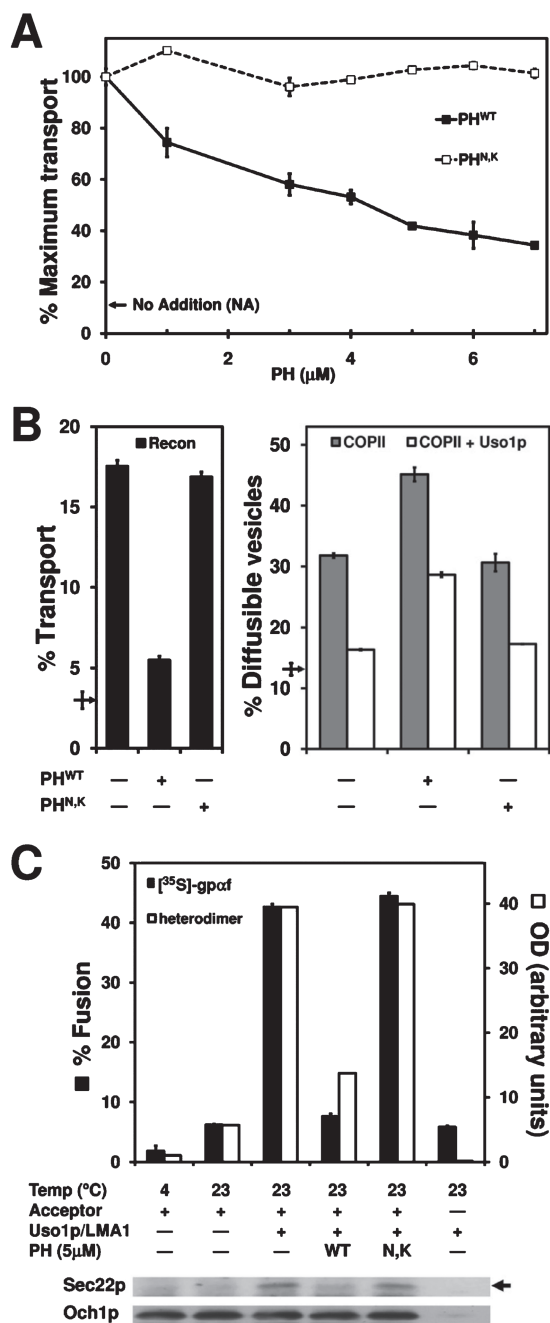
FIGURE 2: PH^{WT} inhibits COPII vesicle fusion with Golgi membranes. (A) Titration of PH^{WT} (black squares) and PH^{N,K} (white squares) in one-stage transport experiments was performed as described in Figure 1A. Maximum transport was equivalent to 20% overall ER-to-Golgi transport. NA represents background level of transport in the absence of transport factors. (B) Overall transport (black bars), budding (gray bars) and tethering (white bars) assays were carried out simultaneously as in Figure 1. PH^{WT} and PH^{N,K} were tested at 5 µM. Arrows show background transport in the absence of transport factors. (C) Fusion reactions were conducted as in Figure 1B and monitored for the formation of cross-linked Bet1p^{I83C}-Sec22p^{D153C} heterodimers (white bars and immunoblot) and for Golgi modification of [^{35}S]gpαf (black bars). Reactions were probed with antibodies against Sec22p and Och1p (loading control). Arrow indicates position of cross-linked linked Bet1p^{I83C}-Sec22p^{D153C} heterodimer.

formation of disulfide cross-links between cognate SNARE protein pairs. In this assay, the SNARE proteins Bet1p and Sec22p contain cysteine residues introduced into their SNARE domains in positions

that produce cross-linked adducts when assembled into SNARE complexes (Flanagan and Barlowe, 2006). Briefly, we performed two-stage fusion reactions chasing Bet1p^{I83C} vesicles into Sec22p^{D153C} acceptor membranes and monitored formation of the Bet1p-Sec22p disulfide cross-linked heterodimer in parallel with transport of [^{35}S] gpαf. As shown in Figure 2C, the inhibition of gpαf transport by 5 µM PH^{WT} mirrors the inhibition of SNARE complex formation, whereas 5 µM PH^{N,K} did not cause detectable inhibition in this assay. Taken together, these data indicate that the PH^{WT} domain inhibits fusion of COPII vesicles through specific interactions with PI(4)P and suggests that the role of PI(4)P in fusion is upstream or simultaneous with *trans*-SNARE complex assembly.

To further explore these results, we sought to biochemically deplete PI(4)P by treating membranes with recombinant Sac1, a phosphoinositide lipid phosphatase that removes the phosphate residue from the inositol head group of PI(4)P. As a control we used the catalytically inactive mutant Sac1^{C392S} (Maehama *et al.*, 2000). Surprisingly, titration of these proteins in two-stage fusion reactions showed that the mutant Sac1^{C392S} was a potent inhibitor of fusion whereas the Sac1^{WT} protein did not display an equal inhibitory effect (Figure 3A). Moreover, in transport, budding, tethering, and two-stage fusion assays, addition of 2.8 µM Sac1^{C392S} inhibited transport, caused COPII vesicles to accumulate, inhibited vesicle fusion, and blocked formation of cross-linked SNARE complexes (Figure 3, B and C). These results are similar to PH^{WT} domain inhibition and suggested that the catalytically inactive mutant Sac1^{C392S} acts as a PI(4)P lipid ligand. To test this hypothesis, we performed liposome-binding assays to monitor binding of Sac1^{WT} and Sac1^{C392S} to PI(4)P liposomes. As shown in Figure 3D, 32% of Sac1^{C392S} bound to PI(4)P liposomes (lane 12) compared to 2% background binding to PI liposomes (lane 10). In contrast, 13% of Sac1^{WT} bound to PI(4)P liposomes (compare lanes 12 and 6) with similar background binding to PI liposomes (compare lanes 10 and 4). Together, these results indicate that the catalytically inactive Sac1^{C392S} mutant inhibits vesicle fusion through tight binding to membrane PI(4)P as observed for the PH^{WT} domain.

We were surprised that Sac1^{WT} treatment in the 5 µM range did not produce stronger inhibition of vesicle fusion. We considered, however, the possibility that our assay conditions may not be optimal for Sac1 phosphatase activity to deplete PI(4)P from semi-intact cell membranes. To promote Sac1^{WT} activity, semi-intact cell acceptor membranes were incubated with higher concentrations of Sac1^{WT} for 30 min at 12°C and then were diluted into standard two-stage fusion reactions. As shown in Figure 4A, pretreatment of acceptor membranes with increasing concentrations of Sac1^{WT} caused a dose-dependent inhibition of vesicle fusion with concentrations greater than 25 µM near the minus acceptor control level. It should be noted that after pretreatment of membranes with the indicated concentration of Sac1^{WT}, membranes were diluted approximately eightfold such that maximal final concentrations of Sac1^{WT} in transport reactions were near those used in Figure 3A. Therefore the inhibitory effects of Sac1^{WT} were likely due to catalytic activity of the enzyme and not due to lipid ligand binding. Finally, pretreatment of acceptor membranes with 50 µM Sac1^{WT} also caused an accumulation of diffusible vesicles in budding reactions and strong inhibition of SNARE complex formation (Figure 4, B and C). These findings suggest that PI(4)P present on acceptor membranes is required for efficient SNARE complex assembly and fusion of ER-derived vesicles with Golgi membranes.

To further support the proposal that Sac1^{WT} inhibition was due to the catalytic phosphatase activity of the enzyme and not due to steric hindrance of binding to PI(4)P, Sac1^{WT}-treated membranes
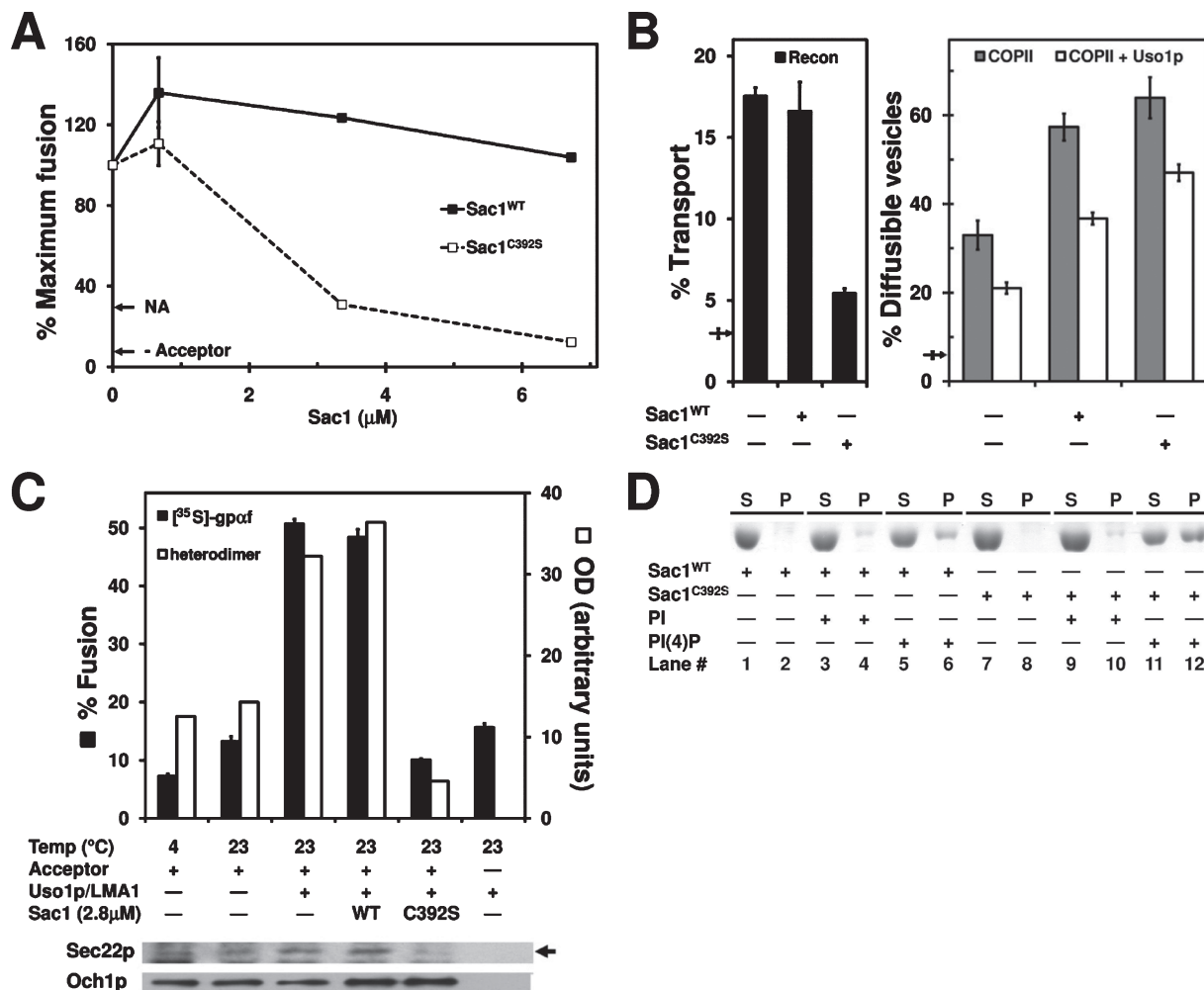
FIGURE 3: Sac1[C392S] inhibits COPII vesicle fusion with Golgi membranes. (A) Titration of Sac1[WT] (black squares) and Sac1[C392S] (white squares) in two-stage fusion reactions was performed as in Figure 1B. Maximum transport was equivalent to 32% fusion. NA shows the background level of fusion in the absence of transport factors and –Acceptor represents fusion level in the absence of added semi-intact cell acceptor membranes. (B) Overall transport (black bars), budding (gray bars), and tethering (white bars) assays were carried out simultaneously as in Figure 2B. Sac1[WT] and Sac1[C392S] were tested at 2.8 μM. Arrows show background transport in the absence of transport factors. (C) Fusion reactions were carried out as in Figure 2C and monitored for the formation of cross-linked Bet1p[I83C]-Sec22p[D153C] heterodimers (white bars and immunoblot) and for Golgi-modified [35S]gpαf (black bars). (D) Sac1[C392S] is a PI(4)P lipid ligand. Sac1[WT] or Sac1[C392S] (3 μM) were incubated with buffer (lanes 1, 2, 7, and 8), PI liposomes (lanes 3, 4, 9, and 10), or PI(4)P liposomes (lanes 5, 6, 11, and 12) for 30 min at 30°C. Liposomes were recovered by sedimentation, and the amount of protein in the supernatant (S) or co-sedimenting with liposomes in the pellet (P) was determined by Coomassie blue staining of 7.5% SDS–PAGE gels. Liposome molar composition: 50% PC, 30% PE, 10% PS, and 10% PI or PI(4)P.

were washed prior to two-stage fusion reactions to remove excess Sac1[WT]. As shown in Figure 4D, this wash did not substantially reduce transport in mock treated membranes (washed vs. unwashed), whereas the 50 μM Sac1[WT] pretreatment inhibited transport to near the minus acceptor control level and was not rescued by the wash. To show that the wash procedure was effective, immunoblot analysis documented that ~76% of Sac1[WT] was removed after washing treated semi-intact cell acceptor membranes (Figure 4D). Moreover, we observed that formation of the Bet1p[I83C]-Sec22p[D153C] cross-linked heterodimer was strongly inhibited by Sac1[WT] pretreatment and was not rescued by the wash (Supplemental Figure S1A). In addition, direct measurement of PI(4)P levels in semi-intact cell membranes demonstrated that the Sac1[WT] pretreatment was effective. After control incubations, semi-intact cell membranes contained 123 pmol PI(4)P/10[9] cells, whereas Sac1[WT]-treated membranes

contained 78 pmol PI(4)P/10[9] cells (Supplemental Figure S1B). Finally, we found that N-ethylmaleimide inactivation of Sac1[WT] (Maehama et al., 2000) completely prevented its inhibitory activity in two-stage fusion reactions compared to mock treated Sac1[WT] (Supplemental Figure S1C). Based on the collective evidence, we conclude that pretreatment of membranes with 50 μM Sac1[WT] inhibits vesicle fusion through reduction of membrane PI(4)P levels.

The fact that preincubation of acceptor membranes with Sac1[WT] inhibited transport (Figure 4) suggested that PI(4)P is required on Golgi membranes. PI(4)P, however, may also be required on COPII vesicles for fusion with the Golgi compartment. To address this issue, we took advantage of the fact that budding is not inhibited by treatment with 50 μM Sac1[WT] (Figure 4B). For this experiment, microsomes containing [35S]gpαf were treated with 50 μM Sac1[WT] or buffer for 30 min at 12°C. Treated microsomes were then used to
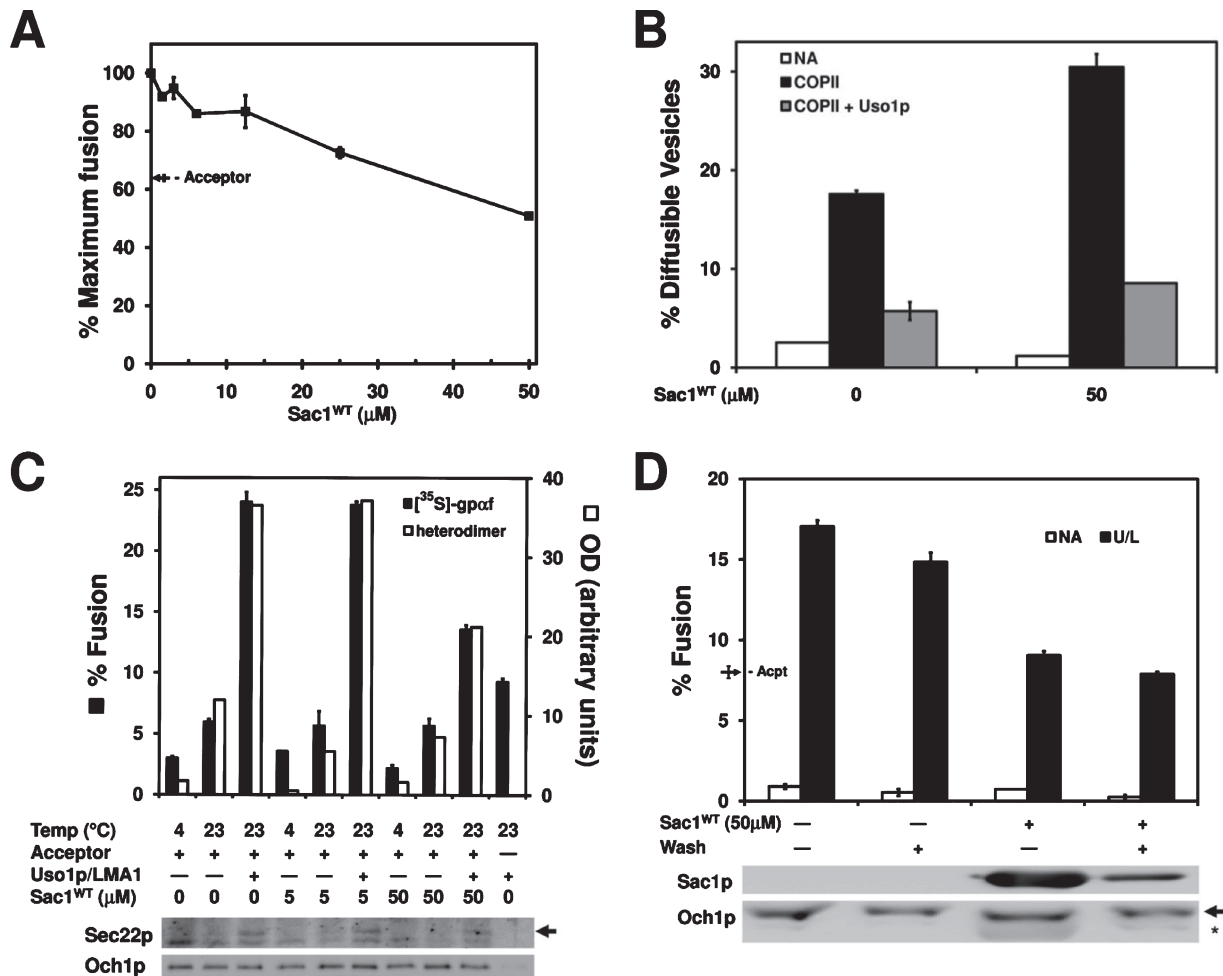
FIGURE 4: Pretreatment of membranes with 50 μM Sac1^{WT} inhibits COPII vesicle fusion with Golgi membranes. (A) Semi-intact cell membranes pretreated with Sac1^{WT} at 12°C for 30 min were used in two-stage fusion reactions as described in Figure 1B. Maximum transport is equivalent to 35% fusion. (B) Semi-intact cells pretreated with 50 μM Sac1^{WT} as in panel A were used in budding (black bars) and tethering (gray bars) assays as in Figure 1C. NA (white bars) shows background budding in the absence of transport factors. (C) Sec22p^{D153C} semi-intact cells were pretreated with 50 μM Sac1^{WT} as in panel A, and then fusion reactions were carried out as in Figure 2C monitoring the formation of cross-linked Bet1p^{I83C}-Sec22p^{D153C} heterodimers (white bars and immunoblot) and Golgi-modified [^{35}S]gpαf fusion (black bars). (D) Reactions were assembled as in panel A and washed twice with excess buffer prior to setting up two-stage fusion reactions. NA shows background fusion in the absence of fusion factors, and the arrow indicates background fusion in the absence of acceptor membranes. Aliquots of the treated membranes were immunoblotted for Sac1p using an anti-penta-His antibody and for the Golgi membrane protein Och1p (loading control). Arrow indicates Och1p; asterisk indicates a cross-reactivity of anti-Och1p with the Sac1^{WT} protein.

generate COPII vesicles, and vesicles were purified by flotation on Nycodenz density gradients (Barlowe, 1997). In two-stage fusion reactions, floated vesicles containing [^{35}S]gpαf were chased into untreated acceptor membranes. Notably, vesicles generated in the presence of 50 μM Sac1^{WT} chased into acceptor Golgi membranes as efficiently as control vesicles (Figure 5), indicating that PI(4)P is required only at the Golgi compartment for fusion with COPII vesicles. Together these findings demonstrate that PI(4)P is required on Golgi acceptor membranes for *trans*-SNARE complex assembly and subsequent fusion with COPII vesicles.

ER-Golgi fusion factors in PI(4)P-dependent vesicle fusion
We hypothesized that PI(4)P on Golgi membranes interacted with protein fusion factors to mediate bilayer fusion. If PI(4)P recruits a cytosolic factor for COPII vesicle fusion with Golgi membranes, addition of crude cytosol might be expected to reverse PH^{WT} inhibition.

We performed two-stage fusion reactions with increasing concentrations of cytosol in the presence of 3, 5, or 7 μM PH^{WT}. High concentrations of cytosol, however, did not rescue PH^{WT} inhibition of COPII vesicle fusion with Golgi membranes (Supplemental Figure S2) suggesting that such a PI(4)P binding fusion factor was not present in our cytosol preparation.

Next, we treated semi-intact cells with inhibitory concentrations of PH^{WT}, Sac1^{WT}, or Sac1^{C392S} and examined if known proteins involved in vesicle tethering and fusion were displaced from the membranes. We did not detect differences in membrane association of Ypt1p, Uso1p, Sly1p, or subunits of the conserved oligomeric Golgi (COG) and TRAPP complexes. In contrast, low levels of the SNARE disassembly chaperone Sec18p and the Grh1/Bug1 tethering complex were detected in the supernatant fraction after treatment of semi-intact cell membranes (Supplemental Figure S3). This observation suggested that PI(4)P levels influence membrane
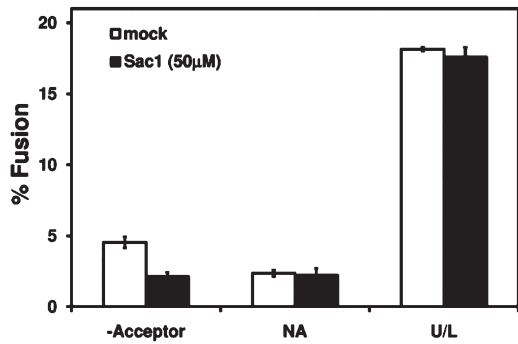
FIGURE 5: PI(4)P is required at the Golgi for fusion with COPII vesicles. Microsomes containing [^{35}S]gpαf were treated with 50 μM Sac1^{WT} or buffer (mock) at 12°C for 30 min. Treated microsomes were then used to generate COPII vesicles, and vesicles were purified by flotation on Nycodenz density gradients. Isolated vesicles were then incubated in two-stage fusion reactions with untreated semi-intact cell acceptor membranes as in Figure 1B.

association of specific fusion factors. We note, however, that addition of a crude cytosol, which contained Grh1p, Bug1p, and Sec18 (Supplemental Figure S2), or the addition of purified Sec18p (see Figure 7 later in the paper) did not compete with PH^{WT} for inhibition of transport.

To further explore the hypothesis that a PI(4)P binding factor promotes COPII vesicle fusion, we took a candidate-based approach to screen viable yeast deletion strains lacking proteins shown to have affinity for PI(4)P (Supplemental Table S1). Previous reports have characterized PI(4)P binding proteins in yeast based on an analysis of PH domain–containing proteins (Yu et al., 2004) and on lipid-binding assays using proteome chips (Zhu et al., 2001). We prepared semi-intact cell acceptor membranes from more than 20 different candidate strains, including the combined osh1Δ osh2Δ osh3Δ deletion mutant (Beh et al., 2001) to account for potential redundancy. In two-stage fusion reactions, all of the acceptor membranes tested displayed wild-type levels of fusion with the exception of gcs1Δ strain, which was reduced to 47% of the wild-type level (Supplemental Table S1). GCS1 encodes an Arf-GAP known to act in COPI-dependent Golgi to ER retrograde transport but is also reported to regulate assembly of ER-Golgi SNARE protein complexes (Poon et al., 1999; Schindler and Spang, 2007). Further experimentation will be required to determine whether the gcs1Δ mutation produces a direct or indirect effect on fusion of COPII vesicles with Golgi membranes. We note, however, that addition of crude cytosol to transport reactions, which contained Gcs1p, did not reverse PH^{WT} inhibition of transport (Supplemental Figure S2).

We also considered the possibility that essential membrane-bound proteins could be involved in PI(4)P-dependent vesicle fusion at Golgi membranes. The ER-Golgi SNARE proteins could interact with PI(4)P as suggested by studies of other SNARE-dependent membrane fusion events (James et al., 2008; Lam et al., 2008; Mendonsa and Engebrecht, 2009; Mima and Wickner, 2009; Murray and Tamm, 2009). To investigate this hypothesis we characterized PI(4)P-dependent vesicle fusion in membranes overexpressing Sly1p and the four ER-Golgi SNARE proteins.

Overexpression of SNARE proteins and Sly1p alleviates the PI(4)P requirement in fusion

Because inhibition of PI(4)P function in ER-to-Golgi transport prevented formation of SNARE complexes but did not have a significant effect on vesicle tethering, we hypothesized that the role of

PI(4)P in fusion might be required downstream of tethering but before or simultaneous with SNARE protein function. Thus, we reasoned that overexpression of the fusion machinery (all four ER-Golgi anterograde SNAREs and Sly1p) might yield acceptor membranes that are resistant or less sensitive to the PH^{WT} inhibitor. We constructed the overproducer strains by transforming wild-type yeast with empty vectors (control) or with 2μ plasmids containing the BET1, BOS1, SEC22, SED5, and/or SLY1 under control of their endogenous promoters. Different combinations of SNARE overexpressing acceptor membranes were tested in two-stage fusion reactions. Overexpression of certain combinations produced modest levels of resistance to 3 or 5 μM PH^{WT} (unpublished data). Overexpression of all four anterograde ER-Golgi SNAREs and SLY1, however, provided a higher level of resistance to PH^{WT} compared to the partial overexpressor strains. Therefore we further characterized this overexpressor strain.

To determine fold overexpression and test whether overexpression influenced other ER-to-Golgi transport factors, we prepared semi-intact cell membranes from the strain overexpressing BET1, BOS1, SEC22, SED5, and SLY1 (from here on referred to as the overexpressor) for comparison with control strains. Experiments to assess the distribution of proteins contained in total, soluble, and membrane pellet fractions monitored the overexpressed proteins and a variety of other ER- and Golgi-localized markers (Figure 6A). We observed that Bet1p, Bos1p, Sec22p, Sed5p, and Sly1p were overexpressed three- to ninefold (compare total lanes), whereas the expression level and fractionation behavior of other marker proteins was not detectably altered.

Bet1p, Bos1p, Sec22p, and Sed5p are known to cycle between the ER and Golgi compartments and are efficiently packaged into COPII vesicles (Cao and Barlowe, 2000); therefore, we expected increased levels of these SNARE proteins in COPII vesicles from
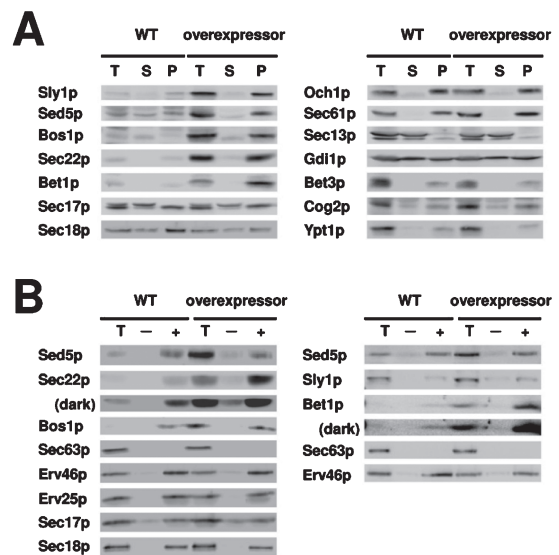

FIGURE 6: Characterization of membranes that overexpress anterograde ER-Golgi SNARE proteins and Sly1p. (A) Semi-intact cells from the overexpressor strain containing 2μ-BET1, 2μ-BOS1, 2μ-SEC22, 2μ-SED5, and 2μ-SLY1 (CBY3061) and the wild-type strain (CBY3062) were fractionated into soluble (S100) and pellet (P100) fractions for immunoblot analysis. (B) Budding reactions in which CBY3061 and CBY3062 microsomes were incubated in the absence (–) or presence (+) of COPII proteins for 30 min at 23°C. Immunoblots compare indicated proteins in budded vesicle fractions with one-tenth of total (T) budding reactions. Longer exposures (dark) are included for the Sec22p and Bet1p immunoblots.

overexpressor membranes. Relative COPII packaging efficiencies were measured in budding assays using microsomes from the wild type and overexpressor strains. As shown in Figure 6B, the ER-Golgi SNARE proteins were 8- to 15-fold more abundant in overexpressor microsomes compared to wild type (total lanes). In budding assays, we observed increased levels of Bet1p (1.5-fold), Bos1p (2-fold), and Sec22p (5-fold) in COPII vesicles. Sed5p and Sly1p, however, were not increased in vesicles, which may be explained by the steady-state localization of Sed5p to *cis*-Golgi membranes (Wooding and Pelham, 1998). As a negative control, the ER resident Sec63p was not packaged into COPII vesicles. The packaging of known COPII vesicle proteins, such as Erv25p and Erv46p, was not changed. Taken together, these data show that COPII vesicles from overexpressor membranes contain more Bet1p, Bos1p, and Sec22p than wild-type membranes and that the distribution of other proteins involved in ER-to-Golgi transport was not sensitive to overexpression of Sly1p and the anterograde ER-Golgi SNARE proteins.

We next investigated how this overexpression condition influenced vesicle fusion. Addition of Sec18p to one-stage and two-stage fusion reactions has a minor effect on fusion rates, probably because of the abundant levels of Sec18p found in association with semi-intact cell membranes (Barlowe, 1997). We reasoned, however, that overexpressor membranes might require additional Sec18p to prime SNARE proteins for maximal fusion. Titration of recombinant Sec18p in one-stage transport reactions using overexpressor membranes indicated that ~4 μM produced maximal stimulation (unpublished data), similar to previous titrations of Sec18p using washed Golgi membrane preparations (Barlowe, 1997). As shown in Figure 7A, we observed that transport with overexpressor and control membranes was indistinguishable in the absence of Sec18p. Addition of 4 μM Sec18p, however, stimulated transport ~1.6-fold in overexpressor membranes, whereas wild-type membranes were not stimulated by Sec18p addition. Moreover, there was a decrease in sensitivity to inhibition by 3 μM PH^{WT} in the overexpressor membranes compared to control, which was further enhanced by addition of 4 μM Sec18p. Interestingly, the level of transport in the overexpressor membranes in the presence of Sec18p and PH^{WT} was similar to that of wild-type membranes in the absence of PH^{WT} (Figure 7A).

Sec18p likely stimulates transport at the vesicle fusion stage by resolving abundant *cis*-SNARE complexes in overexpressor membranes, although there could be an influence on vesicle budding. To test this possibility, we performed [^{35}S]gpαf budding reactions from wild type and overexpressor microsomes and found that there were no significant differences in gpαf budding between the wild type and overexpressor membranes with or without added Sec18p (Figure 7B). These data indicate that Sec18p treatment potentiates fusion of COPII vesicles and that transport in the presence of 4 μM Sec18p with the overexpressor membranes is significantly less sensitive to the PH^{WT} inhibitor.

Finally, acceptor membranes from the overexpressor strain were used in two-stage fusion experiments using wild-type vesicles in the presence or absence of recombinant Sec18p and increasing concentrations of PH^{WT}. We observed that PH^{WT} produced the expected dose-dependent inhibition of transport when chasing wild-type vesicles into wild-type membranes (Figure 7C, compare to Figure 2A) and that addition of Sec18p to this reaction displayed partial rescue of the PH^{WT} inhibition. Interestingly, transport was significantly less sensitive to increasing concentrations of PH^{WT} inhibitor when wild-type vesicles were chased into overexpressor membranes, and the addition of Sec18p significantly enhanced this rescue (Figure 7C and Supplemental Figure S4). Together, these data
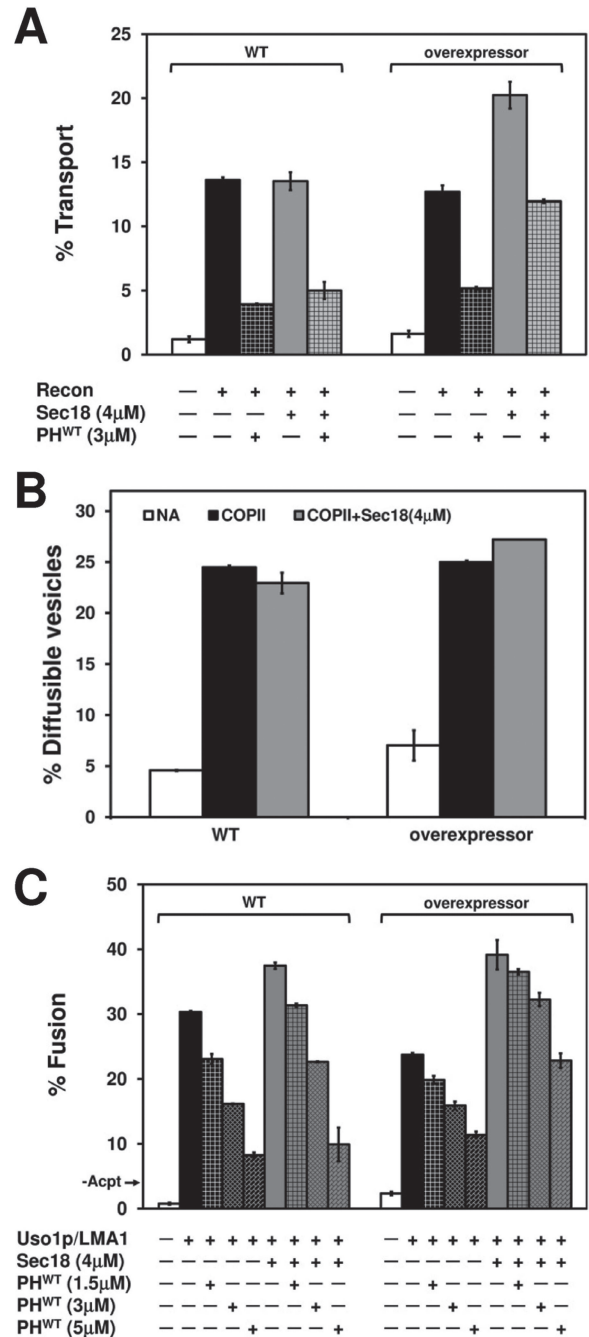

FIGURE 7: Overexpression of anterograde ER-Golgi SNAREs and Sly1p partially rescues PH^{WT} inhibition. (A) Transport in wild-type (CBY3062) and overexpressor (CBY3061) semi-intact cells incubated for 20 min at 4°C with 3 μM PH^{WT} and/or 4 μM Sec18p followed by transport for 1 h at 23°C. (B) Microsomes treated as in panel A were mock treated (NA) or incubated with COPII proteins for 30 min at 23°C to monitor budding. (C) Indicated semi-intact cell acceptor membranes were pretreated with PH^{WT} and/or 4 μM Sec18p for 20 min at 4°C, then were incubated with COPII vesicles containing [^{35}S]gpαf in the presence of fusion factors for 1 h at 23°C. The −Acpt condition indicates background fusion in the absence of added acceptor membranes. The data in panel C are plotted relative to control transport levels in Supplemental Figure S4.

indicate that increasing the amount of anterograde ER-Golgi SNARE proteins and Sly1p does not bypass but alleviates the requirement for PI(4)P on Golgi membranes for vesicle fusion.

PI(4)P is required for ER-to-Golgi transport in vivo

A central role for PI(4)P in Golgi secretory function was first reported in *sec14* and *pik1* mutants, which show reduced levels of cellular PI(4)P and kinetic defects in Golgi transport at restrictive temperatures (Hama *et al.*, 1999; Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000). At steady state, Pik1p localizes to *trans*-Golgi and inner-nuclear compartments (Walch-Solimena and Novick, 1999; Strahl *et al.*, 2005). In both yeast and mammalian cells, Pik1p-generated Golgi PI(4)P is required to recruit clathrin adaptor proteins for export from *trans*-Golgi membranes (D'Angelo *et al.*, 2008; Clague *et al.*, 2009). Therefore a reduction in PI(4)P at Golgi membranes caused by shifting *pik1* mutants to the restrictive temperature strongly inhibits anterograde transport from the Golgi compartment. It should be noted, however, that ER-to-Golgi transport in these experiments was also kinetically delayed (Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000).

On the basis of these reports and our in vitro data, we hypothesized that a pool of Golgi-localized PI(4)P is required for proper trafficking into *cis*-Golgi compartments as well as out of the *trans*-Golgi complex. We also considered the possibility that a PI(4)P gradient across the Golgi complex, with highest requirements at

trans-compartments, could cause inhibition of *trans*-Golgi export first, followed by inhibition of ER-to-Golgi transport. To test this hypothesis, we modified the protocol from Audhya and colleagues to increase the amount of time mutant cells were incubated at the restrictive temperature prior to initiating the pulse-chase experiment (Audhya *et al.*, 2000). As shown in Figure 8A, *pik1–83* cells displayed a dramatic block in maturation of the ER-form (p1) of carboxypeptidase Y (CPY) into the Golgi-form (p2) compared to wild-type cells, indicative of a block in ER-to-Golgi transport. Taken together, these results suggest that traffic out of the *trans*-Golgi complex is more sensitive to PI(4)P depletion (Audhya *et al.*, 2000) but that continued deficiency in Pik1p activity blocks transport to the *cis*-Golgi compartment. In addition, it is also possible that PI(4)P could be required for additional transport steps within the Golgi complex.

Given the highly dynamic nature of the Golgi complex, we investigated an alternative possibility that loss of Pik1p caused a general disassembly of Golgi structures. To address this possibility, we compared growth rates and Golgi morphologies in *pik1–83* and wild-type cells after shift to the restrictive temperature. As shown in Figure 8B, both strains displayed similar growth kinetics and remained metabolically active up to 1 h after the shift to 37°C. At the
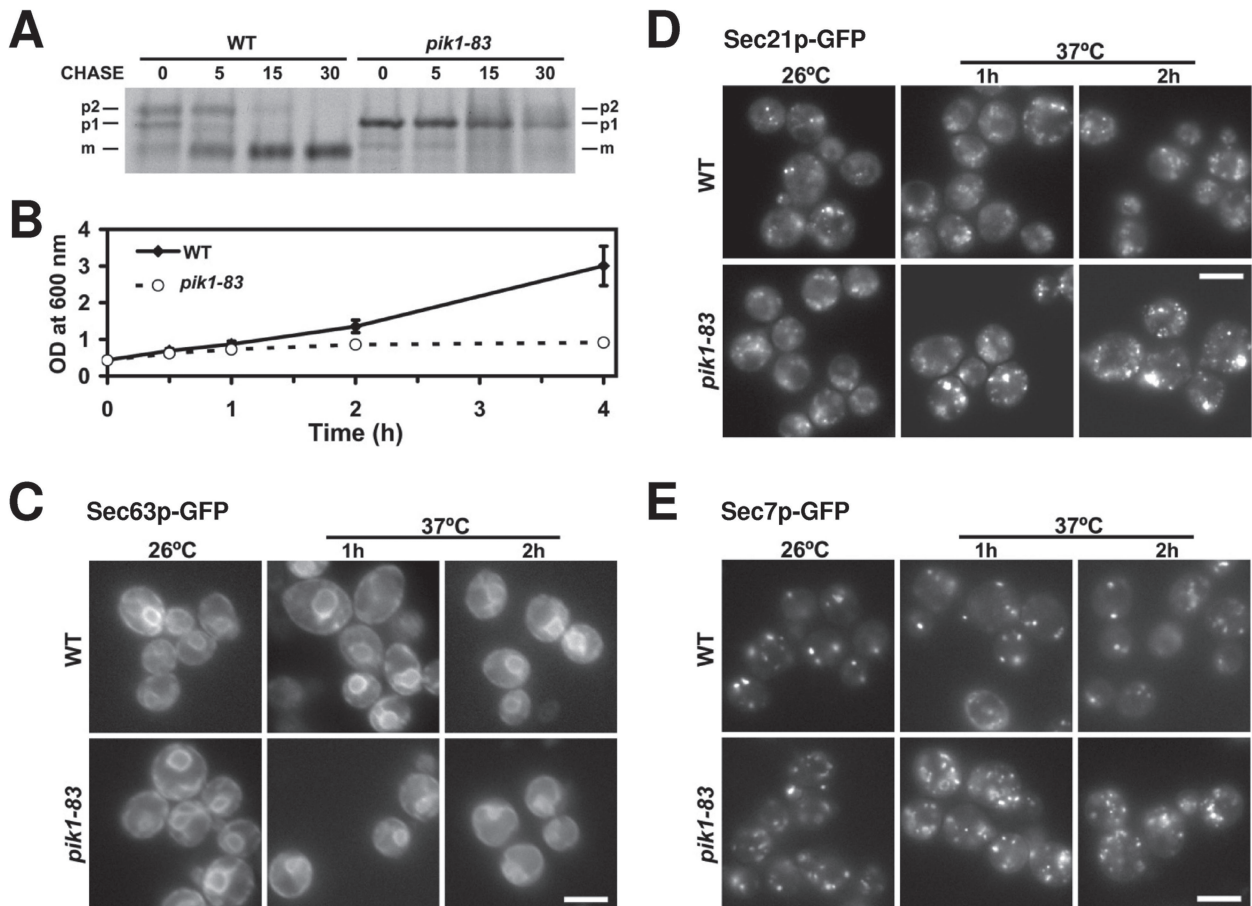

FIGURE 8: Depletion of PI(4)P in vivo causes a block in ER-to-Golgi transport and influences Golgi morphology. (A) Wild-type (SEY6210) and *pik1–83* (AAY104) strains grown to a mid-log stage at 26°C were shifted to 37°C for 45 min, pulsed for 7 min at 37°C, and chased at 37°C for the indicated times. The ER (p1), Golgi (p2), and vacuolar (m; mature) forms of CPY are indicated. Note the block in maturation of p1 CPY in the mutant. (B–E) Wild-type (CBY2970, CBY2973, CBY2974) and *pik1–83* (CBY2980, CBY2983, and CBY2984) cells expressing Sec63p-GFP (C), Sec21p-GFP (D), or Sec7p-GFP (E) were grown on YPD at 26°C to 0.4 OD$_{600}$/ml and shifted to 37°C. (B) Cultures were analyzed for growth over time by absorbance. (C-E) *pik1–83* cells retain ER (C), *cis*-Golgi (D), and late-Golgi (E) structures. Scale bars: 5 µm. A quantitative analysis of ER and Golgi morphologies observed in a population of cells at the 1-h time point is provided in Supplemental Figure S5.

2- and 3-h time points, however, the *pik1–83* cells ceased growth. We note that our chase was initiated at a time point that roughly corresponded to the last chase time point of the published data (Audhya *et al.*, 2000) and that the 5- and 15-min time points in our pulse-chase experiment corresponded to a total time at the restrictive temperature of 57 and 67 min, respectively, suggesting that the observed block in ER-to-Golgi transport was not caused by a general metabolic defect.

To inspect organelle morphologies after the temperature shift, we monitored ER, *cis*-Golgi, and *trans*-Golgi markers by fluorescence microscopy of Sec63p-GFP, Sec21p-GFP, and Sec7p-GFP, respectively. As shown in Figure 8, C–E, ER and Golgi structures were visible in mutant cells after 1 h and even 2 h at the restrictive temperature. Based on the time frame of our pulse-chase experiment, we quantified morphologies 1 h after shift to the restrictive temperature. ER morphology was not significantly altered at the 1-h time point as observed by a ~15% reduction in the number of cells with wild-type structures (Figure 8C and Supplemental Figure S5C). In addition, we did not detect noticeable alterations in ER exit site morphology when Sec13p-GFP was observed in *pik1–83* cells compared to wild type (unpublished data). Both *cis*- and *trans*-Golgi membrane markers, however, showed an increase in the number of smaller Golgi puncta accompanied by larger ring-like structures that presumably correspond to Berkeley bodies also observed in *arf1Δ*, *gea1Δ*, *sec7–1*, and *pik1–101* mutants (Gaynor *et al.*, 1998; Peyroche *et al.*, 2001; Bruinsma *et al.*, 2004; Karhinen and Makarow, 2004; Demmel *et al.*, 2008). Quantification of Golgi morphologies demonstrated acute defects at *trans*-Golgi compartments with a ~64% reduction the number of cells with wild-type *trans*-Golgi structures and moderate defects at *cis*-Golgi compartments with a ~36% reduction in the number of cells with wild-type *cis*-Golgi structures (Figure 8, D and E, and Supplemental Figure S5C). These observations indicate that Pik1p deficiency does not cause a general loss of Golgi membranes and is consistent with a block in both export from *trans*-Golgi compartments and transport into *cis*-Golgi compartments.

DISCUSSION

The study of anterograde ER-to-Golgi traffic has yielded a comprehensive list of proteins and mechanisms involved in vesicle budding, cargo sorting, and fusion of COPII vesicles with the Golgi complex. We know little, however, about the role that specific lipids play in these processes. To advance our understanding, we performed the first screen for phosphoinositide requirements in anterograde ER-to-Golgi transport in yeast using a number of lipid-binding domains, enzymes, and enzyme inhibitors in cell-free budding, tethering, and fusion assays. We classified the effects of these compounds into distinct categories. The first group comprised the PI(4,5)P$_2$ binding domains (ENTH and MED), which did not have a significant effect on ER-to-Golgi transport. A second group comprised inhibitors that targeted both budding and fusion and included the PLC inhibitor U73122, a PI-specific PLC, and the PI(3)P binding domain (FYVE). A third group comprised inhibitors that specifically targeted fusion of COPII vesicles and included the PI(4)P and DAG binding domains (PH and C1b, respectively).

PI(4)P is the most abundant cellular phosphoinositide (Lemmon, 2008) and is prevalent at the Golgi complex with additional pools detected at the plasma and nuclear membranes (D'Angelo *et al.*, 2008). In both yeast and mammalian cells, the Golgi pool of PI(4)P is required for Golgi secretory function (D'Angelo *et al.*, 2008), and was recently shown to be required for retention of *cis*-, medial-, and *trans*-Golgi glycosyltransferases in yeast (Tu *et al.*, 2008; Wood

et al., 2009). Golgi levels of PI(4)P are thought to be regulated by multiple activities, including the PI-transfer protein Sec14p (Mousley *et al.*, 2007), the *trans*-Golgi localized PI 4-kinase Pik1p, and the ER-localized phosphoinositide-phosphatase Sac1p (Mayinger, 2009). Using a PI(4)P-specific PH domain lipid ligand and a recombinant Sac1p-phosphatase to reduce the levels of PI(4)P in vitro, we observed that PI(4)P was not involved in budding or tethering of COPII vesicles, but was required on Golgi acceptor membranes for fusion with COPII vesicles. More specifically, we found that inhibition of PI(4)P function reduced formation of the SNARE complexes that catalyze fusion of vesicles with Golgi membranes.

In vitro COPII vesicle budding from synthetic liposomes was shown to require low concentrations of PI(4)P and PI(4,5)P$_2$ to achieve efficient binding of Sec23/Sec24 and to support vesicle budding (Matsuoka *et al.*, 1998). Our data indicate that neither phosphoinositide is required for COPII vesicle budding on ER membranes, although other acidic phospholipids on the ER surface could satisfy this requirement. Alternatively, recruitment of Sec23/Sec24 by protein cargo molecules on the surface of ER membranes may be sufficient to assemble COPII coats for vesicle budding, as observed on synthetic proteoliposomes (Sato and Nakano, 2005).

In mammalian cells, evidence suggests that PI(4)P is required for remodeling ER exit sites and regulating COPII vesicle budding (Blumental-Perry *et al.*, 2006). In vitro, we did not detect a requirement for PI(4)P in COPII budding from yeast ER, and in vivo *pik1–83* yeast cells shifted to a restrictive temperature did not accumulate ER membranes or cause disassembly of Golgi compartments as would be expected if COPII budding were inhibited (Wooding and Pelham, 1998; Prinz *et al.*, 2000; Heidtman *et al.*, 2003; Kamena *et al.*, 2008; Lorente-Rodriguez *et al.*, 2009). This contrast may be explained by differences in the structural organization of ER export sites between yeast and mammalian cells. We speculate, however, that PI(4)P functions in both yeast and mammalian cells for fusion of COPII vesicles with Golgi membranes based on the evidence that, in both species, Golgi PI(4)P is required for Golgi secretory function (D'Angelo *et al.*, 2008) and that Golgi PI(4)P levels are tightly regulated by PI 4-kinases and PI(4)P 4-phosphatases in response to growth conditions (Mayinger, 2009). In addition, PI(4)P was recently observed to significantly colocalize with *cis*-Golgi tethering factors in mammalian cells (Hammond *et al.*, 2009). Further experimentation will be needed, however, to demonstrate whether PI(4)P mediates fusion at early Golgi compartments in mammalian cells.

On the basis of the collective findings, we hypothesize that a trafficking-dependent gradient of PI(4)P is established across the Golgi complex with its highest levels at the *trans*-Golgi and lowest levels at the *cis*-Golgi compartments. This PI(4)P gradient would be necessary for transport into and out of the Golgi complex. Because Pik1p and Sac1p have been implicated in regulating Golgi PI(4)P levels in yeast and mammalian cells (Mayinger, 2009), we propose that this gradient is regulated by Pik1p and Sac1p activities. This hypothesis is supported by our observation that *pik1–83* cells shifted to a restrictive temperature for 1 h displayed morphological defects in *trans*-Golgi structures, accompanied by moderate defects in *cis*-Golgi structures. In this framework, inactivation of Pik1p would initially inhibit anterograde transport from *trans*-Golgi membranes (Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000). As retrograde transport continues, depletion of PI(4)P from *cis*-Golgi compartments would ultimately inhibit fusion of ER-derived vesicles with Golgi membranes. This proposal is consistent with our analysis of *pik1–83* semi-intact cell membranes in vitro, which did

not display thermosensitive defects in cell-free transport assays (Supplemental Figure S6). By increasing the time of *pik1–83* inactivation compared to published reports (Audhya *et al.*, 2000), we detected a strong block in ER-to-Golgi transport. It is interesting to note that mutations that mislocalize Sac1p to the Golgi also produce kinetic delays in ER-to-Golgi transport in addition to antero-grade transport defects from the Golgi (Schorr *et al.*, 2001; Konrad *et al.*, 2002). Taken together, these data are consistent with an in vivo requirement for PI(4)P in fusion of COPII vesicles with the Golgi apparatus.

What is the molecular function of PI(4)P in fusion? We observed that overexpression of the fusion factors *BET1, BOS1, SEC22, SED5,* and *SLY1* partially rescued PH^WT-mediated inhibition of COPII vesicle fusion with Golgi membranes. This result indicates that PI(4)P may act concomitantly with SNARE protein function. We hypothesize that *cis*-Golgi localized PI(4)P interacts directly with SNARE proteins and increases their local concentrations at specific fusion sites to promote COPII vesicle fusion with the Golgi. There is precedent for this type of association. SNARE proteins are known to associate with membranes through phosphoinositide-binding domains, as is the case for the soluble SNARE Vam7p, which is recruited to the vacuolar membrane by PI(3)P (Cheever *et al.*, 2001; Fratti *et al.*, 2004). Alternatively, SNARE proteins often contain polybasic juxtamembrane regions, which directly interact with negatively charged lipids (i.e., phosphatidic acid and phosphoinositides), as is the case for yeast and mammalian exocytic SNARE proteins. Moreover, these SNARE–lipid interactions are required for secretion and viability (Van Komen *et al.*, 2005; Vicogne *et al.*, 2006; James *et al.*, 2008; Lam *et al.*, 2008; Mendonsa and Engebrecht, 2009; Murray and Tamm, 2009; Williams *et al.*, 2009). Similar interactions between Golgi-localized PI(4)P and specific motifs in ER-Golgi SNARE proteins may be required to catalyze efficient fusion of ER-derived vesicles with Golgi membranes.

In summary, this study establishes a new requirement for PI(4)P in SNARE-dependent fusion of COPII vesicles with Golgi membranes. Given the level of conservation in ER-to-Golgi transport machinery, PI(4)P function in COPII vesicle fusion with the Golgi complex is likely to be conserved and therefore constitutes an additional mechanism to control transport through the early secretory pathway. A refined cell-free fusion assay and facile genetic approaches should allow us to determine if specific ER-to-Golgi SNARE proteins or potentially other fusion factors are recruited by PI(4)P to membrane fusion sites.

MATERIALS AND METHODS
Yeast strains and media
Yeast strains used in this study are listed in Table 1. Unless noted otherwise, cultures were grown at 26°C (for temperature-sensitive mutant stains) or 30°C in rich medium (YPD: 1% yeast extract, 2% peptone, 2% dextrose) or in minimal medium (YMD: 0.7% yeast nitrogen base without amino acids, 2% dextrose, and appropriate amino acid supplements). Standard yeast (Sherman, 1991) and cloning protocols (Ausubel *et al.*, 1987) were used. Yeast cells were transformed by the lithium acetate method (Elble, 1992).

Plasmids *pRS426-BOS1* and *YEp511-BET1*(*LEU2*) have been described (Dascher *et al.*, 1991; Duden *et al.*, 1994). Plasmid *pRS423-SEC22* was constructed by subcloning a ~1.5-kb *BamHI-EcoR*V fragment of *YEp511-SEC22* (Dascher *et al.*, 1991) into *pRS423* (Christianson *et al.*, 1992). Plasmids *pRS424-SED5* and *pRS327-SLY1* were generated by amplifying BY4742 genomic DNA including 300–500 bp upstream and downstream sequences and cloning into *pRS424* (Christianson *et al.*, 1992) or *pRS327* (Eriksson *et al.*, 2004). Plasmids generated in this study were confirmed by sequencing. FY834 was transformed with appropriate empty vectors or with the *BET1, BOS1, SEC22, SED5,* and *SLY1* 2μ vectors to generate CBY3062 and CBY3061, respectively. Overexpression was confirmed by immunoblot (Figure 6).

Wild-type (SEY6210) and *pik1–83* (AAY104) strains were transformed with *pJK59* [*CEN SEC63-GFP URA3*] (Prinz *et al.*, 2000), or a chromosomal *GFP*x3 tag was introduced into the *SEC21* and *SEC7* loci as described (Rossanese *et al.*, 2001) to generate CBY2970, CBY2973, CBY2974, CBY2980, CBY2983, and CBY2984.

Reagents
Purified lipid-binding domains GST-ENTH (Rosenthal *et al.*, 1999), C1b (C-terminal glutathione S-transferase [GST] cleaved) (Johnson

Strain	Genotype	Source
AAY104	SEY6210; *pik1Δ::HIS3* [*pRS314-pik1–83*]	Audhya *et al.*, 2000
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Brachmann *et al.*, 1998
CBY1584	BY4742; *sec22Δ::KAN^R* [*pRS313-SEC22*(D153C)]	Flanagan and Barlowe, 2006
CBY1676	BY4742; *bet1Δ::KAN^R* [*pRS315-BET1*(I83C)]	Flanagan and Barlowe, 2006
CBY2970	SEY6210; [*pSEC63-GFP, URA3, CEN (pJK59)*]	This study
CBY2973	SEY6210; *SEC21-GFPx3*	This study
CBY2974	SEY6210; *SEC7-GFPx3*	This study
CBY2980	AAY104; [*pSEC63-GFP, URA3, CEN (pJK59)*]	This study
CBY2983	AAY104; *SEC21-GFPx3*	This study
CBY2984	AAY104; *SEC7-GFPx3*	This study
CBY 3061	FY834; [*pRS423-SEC22, HIS3*] [*pRS424-SED5, TRP1*] [*YEp511-BET1, LEU2*] [*pRS426-BOS1, URA3*] [*pRS327-SLY1, LYS2*]	This study
CBY3062	FY834; [*pRS423*] [*pRS424*] [*pRS425*] [*pRS426*] [*pRS327*]	This study
FY834	*MATα his3Δ200 ura3–52 leu2Δ1 lys2Δ202 trp1Δ63*	Winston *et al.*, 1995
SEY6210	*MATα leu2-3,112 ura3–52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al.*, 1988

TABLE 1: Strains used in the study.

et al., 2000), GST-FYVE$_2$ (Gillooly et al., 2000), MED (Wang et al., 2001), HIS$_6$-Sec18p (Thorngren et al., 2004), and U73122 were the gifts of Bill Wickner (Dartmouth).

Plasmids expressing the PH domain of human Fapp1 (PI(4)P adaptor protein-1; residues 1–99) as a wild-type construct (PH^{WT}) (Dowler et al., 2000) or as the W15N, R18K mutant (PH^{N,K}) (Weixel et al., 2008) cloned as N-terminal fusions with maltose binding protein (MBP) were the gifts of Christopher Stroupe (Dartmouth). MBP fusion proteins were purified according to New England Biolabs (Ipswich, MA) recommendations and dialyzed into buffer 88 (20 mM HEPES-KOH pH 7.0, 250 mM sorbitol, 150 mM KOAc, 5 mM MgOAc) without sorbitol. Plasmids expressing the soluble domain of Sac1p (residues 1–507) as a wild-type HIS$_6$-Sac1 construct (Sac1^{WT}) or the catalytically inactive C392S mutant (Sac1^{C392S}) were the gifts of Gregory Taylor (University of Nebraska Medical Center). His$_6$ fusion proteins were purified according to QIAGEN (Valencia, CA) recommendations and dialyzed into buffer 88 without sorbitol containing 0.5 mM dithiothreitol (DTT). The activity of these Sac1 recombinant proteins was confirmed using synthetic liposomes as described (Maehama et al., 2000) and using semi-intact cell membranes (Supplemental Figure S1B).

PI-PLC was purchased from Invitrogen (Carlsbad, CA). Nycodenz, deuterium oxide (D$_2$O), and methylene blue were purchased from Sigma Chemical (St. Louis, MO). Coomassie brilliant blue R-250 was purchased from Bio-Rad (Hercules, CA). All membrane transport inhibitors were diluted with buffer 88 to the appropriate concentration and screened for inhibition of in vitro transport at the indicated concentrations.

Lipids and liposome-binding assay

POPC, POPE, POPS, soy PI (Avanti Polar Lipids, Alabaster, AL), Rhodamine-labeled DHPE (Invitrogen, Carlsbad, CA) and diC16 D-myo-PI(4)P (Echelon Biosciences, Salt Lake City, UT), were purchased from the indicated sources. Liposomes were prepared as described (Patki et al., 1997) with minor modifications. Lipids were mixed in glass tubes at the following molar ratios of 50% POPC, 29.75% POPE, 0.25% Rhodamine labeled DHPE, 10% POPS, and 10% soy PI or diC16 D-myo-PI(4)P, then dried to a thin film under a stream of nitrogen gas and resuspended to a final concentration of 2 mg total lipid / ml Sac1 phosphatase buffer (Maehama et al., 2000): 100 mM NaOAc, 50 mM Bis-Tris, 50 mM Tris-HCl, pH 6.0, 2 mM DTT. Resuspended lipids were sonicated for 5 min at 30°C, separated into 50-µl aliquots, flash-frozen, and stored at −70°C.

Liposome-binding assays were as described (Loyet et al., 1998) with minor modifications. Sac1^{WT} and Sac1^{C392S} proteins were precleared by centrifugation at 270,000 × g for 10 min. Binding reactions (70 µl) containing 3 µM precleared Sac1^{WT} or Sac1^{C392S} protein in Sac1 phosphatase buffer with or without 50 µl of PI or PI(4)P liposomes were incubated for 30 min at 30°C. Bovine serum albumin (BSA) was added to 1 mg/ml to minimize nonspecific binding. Liposomes were recovered by sedimentation at 270,000 × g for 10 min, and an aliquot of the supernatant was collected. Pellets were washed with excess Sac1 phosphatase buffer containing BSA at 1 mg/ml and resuspended to the initial volume with wash buffer. Equivalent volumes were resolved on 7.5% SDS–PAGE gels and stained with 0.05% Coomassie brilliant blue R-250. Densitometric analysis was performed using ImageJ (Rasband, 1997–2005). Fluorometric quantification of Rhodamine labeled DHPE (λex/λem 560 nm/580 nm) indicated that 87% ± 6.7% (SD) of the starting liposomes were recovered in pellet fractions.

Antibodies and immunoblotting

Antibodies against CPY (Rothblatt et al., 1989); Grh1p (Behnia et al., 2007); Kar2p (Brodsky and Schekman, 1993); Och1p, Erv46p (Otte et al., 2001); Sly1p (Flanagan and Barlowe, 2006); Sec22p (Liu and Barlowe, 2002); Bet1p, Bos1p (Sogaard et al., 1994); α1,6-mannose linkages, Sed5p (Cao et al., 1998); Sec63p (Feldheim et al., 1992); Uso1p, Bet3p, Cog2p/Sec35p (Ballew et al., 2005); Erv25p (Belden and Barlowe, 1996); Gdi1p (Garrett et al., 1994); Sec13p (Salama et al., 1993); Ypt1p (Rexach et al., 1994); Sec17p, Sec18p (Haas and Wickner, 1996); and Sec61p (Stirling et al., 1992) have been described. Polyclonal antibodies were raised against full-length untagged Bug1p as follows: Bug1p was cloned into an Impact-CN protein expression vector, purified according to the manufacturer's specifications (New England Biolabs), and used to immunize rabbits by standard procedures (Covance, Denver, PA). For Western blots, anti-Bug1p serum was diluted 1:1,000. Monoclonal anti-5HIS was purchased from QIAGEN. Western blot analysis was performed with nitrocellulose membranes by using the SuperSignal West Pico chemiluminescent substrate (Pierce Chemical, Rockford, IL) and developed with a UVP Bioimaging System (Upland, CA). Densitometric analysis of immunoblots was performed using ImageJ (Rasband, 1997–2005).

In vitro vesicle budding, tethering, and transport assays

Yeast semi-intact cell membranes and ER microsomes were prepared as described (Baker et al., 1988; Wuestehube and Schekman, 1992). Prior to assembling in vitro reactions, semi-intact cell membranes or microsomes were incubated with indicated inhibitors and/or recombinant Sec18p for 20 min at 4°C, or with Sac1^{WT} for 30 min at 12°C as described in the text and figure legends. In vitro assays to measure overall transport, vesicle budding, vesicle tethering, two-stage fusion reactions, and SNARE cross-linking assays using [^{35}S]gpαf were performed as described (Barlowe, 1997; Cao et al., 1998; Flanagan and Barlowe, 2006).

For vesicle purification on Nycodenz gradients, microsomes containing [^{35}S]gpαf were treated with 50 µM Sac1^{WT} for 30 min at 12°C and then used to generate COPII vesicles (Cao et al., 1998). ER-derived vesicles were purified by flotation on Nycodenz density gradients prepared in D$_2$O to strip peripherally associated membrane proteins, and then floated vesicles were chased into washed semi-intact cell membranes at 23°C for 1 h (Barlowe, 1997).

Pulse-chase experiments

Wild type (SEY6210) and pik1–83 (AAY104) strains were grown at 26°C in minimal medium to 0.4 OD$_{600}$/ml (Beckman DU®-64 Spectrophotometer; Fullerton, CA), and then shifted to 37°C for 25 min. These cultures were washed in 37°C-warm pulse-chase medium lacking cysteine, methionine, and sulfate, concentrated to 4 OD$_{600}$/ml (Audhya et al., 2000) and further incubated at 37°C. In total, cultures were shifted to 37°C for 45 min prior to the pulse. Cultures were pulsed for 7 min by the addition of EasyTag EXPRESS^{35}S Protein Labeling Mix (Perkin Elmer-Cetus, Waltham, MA) to a final concentration of ~25 µCi/OD$_{600}$. Cells were chased by the addition of cysteine, methionine, and (NH$_4$)$_2$SO$_4$ at a final concentration of 2.5 mM. Sample collection, lysis, and immunoprecipitations were performed as described (Belden and Barlowe, 1996).

Microscopy

Samples at the indicated time points were concentrated to 14 OD$_{600}$/ml in the presence of 0.008% methylene blue to identify live versus dead cells (Teparic et al., 2004), and 3 ml was applied to slides. Fluorescent and bright field images were acquired using a microscope (model BX51; Olympus) equipped with a 100-W mercury

arc lamp, Plan Apochromat 60× objective (1.4 NA) and a Sensicam QE CCD camera (Cooke Inc., Romulus, MI). The camera and microscope were controlled by the IP Lab system (Scanalytics, Sunnyvale, CA). All images were processed in Openlab software (Improvision, Lexington, MA).

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