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Genetic Analysis of Yeast Sec24p Mutants Suggests Cargo Binding Is Not Co-operative during ER Export

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Many eukaryotic secretory proteins are selected for export from the endoplasmic reticulum (ER) through their interaction with the Sec24p subunit of the coat protein II (COPII) coat. Three distinct cargo-binding sites on yeast Sec24p have been described by biochemical, genetic and structural studies. Each site recognizes a limited set of peptide motifs or a folded structural domain, however, the breadth of cargo recognized by a given site and the dynamics of cargo engagement remain poorly understood. We aimed to gain further insight into the broader molecular function of one of these cargo-binding sites using a non-biased genetic approach. We exploited the *in vivo* lethality associated with mutation of the Sec24p B-site to identify genes that suppress this phenotype when overexpressed. We identified *SMY2* as a general suppressor that rescued multiple defects in Sec24p, and *SEC22* as a specific suppressor of two adjacent cargo-binding sites, raising the possibility of allosteric regulation of these domains. We generated a novel set of mutations in Sec24p that distinguish these two sites and examined the ability of Sec22p to rescue these mutations. Our findings suggest that co-operativity does not influence cargo capture at these sites, and that Sec22p rescue occurs via its function as a retrograde SNARE.

Key words: cargo selection, COPII vesicles, ER export, intracellular traffic, Sec24

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Eukaryotic secretory and membrane proteins are translated on the endoplasmic reticulum (ER) membrane, facilitating translocation across the lipid bilayer and integration of membrane domains. Within the ER, these proteins attain their proper fold, often assisted by the actions of specific chaperones. Once folded, newly synthesized proteins are packaged into transport vesicles that mediate delivery between the ER and Golgi (1,2). A set of cytoplasmic proteins, collectively known as the coat protein II (COPII) coat, generates ER-derived transport vesicles through a well-defined sequence of events. COPII consists of five distinct polypeptides: Sar1p, Sec23p, Sec24p, Sec13p and Sec31p. Vesicle biogenesis is initiated by the small G-protein, Sar1p, which becomes membrane associated when bound to GTP. Sar1p serves both as an initiator of membrane curvature (3) and a molecular switch that recruits the additional COPII proteins (4). The Sec23p/Sec24p heterodimer binds to Sar1p•GTP and this 'pre-budding complex' is the minimal machinery required for recruiting cargo proteins to the nascent vesicle (5,6). Finally, the Sec13p/Sec31p heterotetramer is recruited to form the outer shell of the coat, likely cross-linking adjacent cargo-coat complexes and propagating membrane curvature (7,8).

A central function of the COPII coat is to capture cargo proteins into nascent vesicles for delivery to the Golgi apparatus. Although some proteins may exit the ER via a non-specific process known as 'bulk flow', this avenue of egress is rather inefficient (~1% of a given protein may be captured by stochastic means), and likely contributes in a relatively minor way to protein secretion (9). Conversely, many cargo proteins are markedly enriched in vesicles relative to the donor compartment, and this specificity in capture is largely driven by distinct sorting signals within the amino acid sequence of each protein (10). Some of the sorting signals that promote capture into COPII vesicles have been defined; however, this discovery process is complicated by the sheer volume of diverse traffic that ER-derived vesicles must accommodate: genomic and proteomic analyses carried out in yeast estimate that up to one third of all proteins enter the ER for delivery to various compartments of the secretory pathway (11).

With such a diverse array of client proteins containing a variety of different sorting signals that must be specifically recognized, the COPII coat must incorporate a high degree of flexibility into the process of cargo capture. The key to this remarkably accommodating coat lies with the Sec24p subunit, which functions as a multivalent cargo adaptor (10). A definitive role for Sec24p in cargo capture was established by a combination of structural, biochemical and genetic approaches that identified three independent sites on Sec24p involved in cargo interaction (12,13). These so-called A-, B- and C-sites all recognize distinct ER export motifs and seem to function independently of each other. The A-site recognizes a YxxxNPF motif on the Golgi protein, Sed5p. The B-site binds to three motifs: DxE, LxxLE and LxxME found on Sys1p, Bet1p and Sed5p, respectively. Initially, the C-site was defined by a single amino acid on Sec24p that was required for efficient packaging of the SNARE Sec22p (13). More recently, a crystal structure of mammalian Sec23/Sec24 was solved in complex with Sec22 (14). This new structure confirmed a critical role for

the C-site residue in binding to Sec22, and expanded this site of interaction to include a large surface spanning the interface between Sec23 and Sec24. Importantly, Sec22 interacted with Sec23/24 via a conformational epitope such that only the correctly folded SNARE would be capable of binding.

The utility of all three cargo-binding sites *in vitro* and *in vivo* has been shown by altering key residues at each site (13,15). Specific cargo-packaging defects associated with each mutant form of Sec24p were analyzed by an *in vitro* assay that reconstitutes the formation of ER-derived transport vesicles using purified COPII proteins (16). Furthermore, these mutations had a profound *in vivo* phenotype, rendering cells temperature-sensitive for growth or inviable, highlighting the essential nature of the cargo-capture function of Sec24p. However, despite the detailed mechanistic insight we have into the interaction between Sec24p and a select group of cargo proteins, we lack a complete description of all the cargo proteins that engage the known sites on Sec24p. Furthermore, it remains unclear whether there is cross-talk between separate cargo-binding sites that might function in the ordered recruitment of key cargo proteins. For example, as each vesicle must contain at least one vesicle-associated SNARE, binding of Bet1p at the B-site might trigger an allosteric change in Sec24p such that additional cargo-binding sites would become more readily occupied by secretory cargoes. From the existing crystal structures, no changes in Sec24p conformation were observed upon co-crystallization with cargo peptides, but the possibility remains that in the more complex context of the ER membrane, cargo binding at one site may influence (positively or negatively) cargo recruitment to a second site.

To gain further insight into the molecular interactions that drive export out of the ER, we aimed to use a non-biased genetic approach to identify novel candidate cargo proteins that interact with a key cargo-binding site on Sec24p. We exploited the severe *in vivo* phenotype associated with mutation of the B-site, which is known to bind multiple cargoes, to identify proteins that, when overexpressed, overcome the lethality associated with defective cargo capture. We identified three suppressing genes: *BET1*, *SEC22* and *SMY2,* which correspond to both general (*SMY2*) and specific (*BET1, SEC22*) suppressors. Through the generation of novel alleles of *SEC24*, we investigated the mechanism of *SEC22* suppression and specifically addressed whether cargo engagement at the B-site might be influenced by binding of Sec22p to the C-site. Our findings suggest that there is no cross-talk between the B- and C-sites and instead suggest that enhanced retrograde traffic improves the viability of cells expressing the B-site mutant form of Sec24p.

Results

Mutation of key residues in each of the three cargo-binding sites on Sec24p yields proteins that remain functional in terms of generating COPII vesicles *in vitro*, albeit with a reduced set of cargo proteins (13,15).

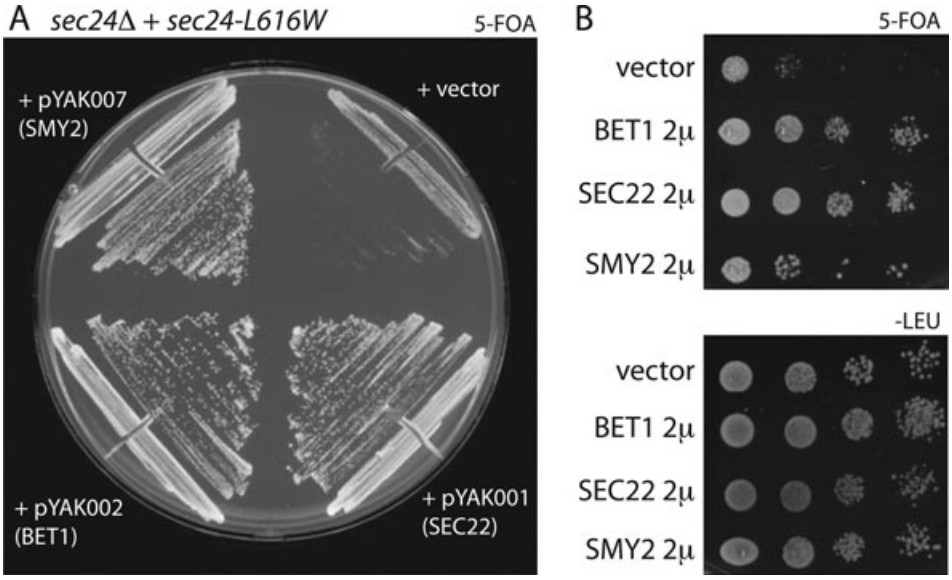

Figure 1: Identification of multicopy suppressors of a *SEC24* cargo-binding mutant. A) A strain containing a deletion in the chromosomal copy of *SEC24* and expressing wild-type *SEC24* from a *URA3*-marked plasmid and sec24-L616W from a *HIS3*-marked plasmid was transformed with either empty vector or the candidate suppressing plasmids as indicated. Cells were streaked onto medium containing 5-FOA to counterselect for the *URA3*-marked wild-type version of Sec24p. As Sec24-L616W cannot support viability on its own, only strains co-expressing suppressing genes were able to grow. B) Individual candidate suppressor genes were subcloned into a 2μ vector and retested for their ability to suppress the lethality of the Sec24-L616W mutant on 5-FOA (top panel).

These cargo-export deficits render cells inviable under certain conditions: the most severe mutants cannot complement the loss of Sec24p, whereas other mutants show *in vivo* complementation that is dependent on the Sec24p homolog, Iss1p/Sfb2p. We sought to use this *in vivo* lethality to identify suppressors of the Sec24p B-site mutants that might be candidate cargo proteins. We reasoned that increasing the abundance of specific cargo proteins that are impaired in their association with this site will promote their capture into vesicles, either by stochastic sampling of the ER membrane during vesicle biogenesis (i.e. bulk flow) or by increasing the apparent affinity of the cargo for a weakened but partially functional binding site (i.e. mass action). Previous work has shown that increased gene dosage of a specific B-site cargo protein, the pleiotropic drug transporter Yor1p, can overcome the oligomycin sensitivity associated with Sec24p B-site mutants (17).

Isolation of multicopy suppressors of the Sec24p B-site

To search for novel cargo proteins that were able to rescue Sec24p cargo-binding site mutants, we used an overexpression approach to isolate genes that complement the viability of a *sec24*Δ strain expressing a Sec24p B-site mutant, *sec24-L616W*. *sec24-L616W* is a particularly severe mutation that is unable to complement the loss of *SEC24*, even in the presence of *ISS1* (13). Viability of this strain is maintained by the presence of a wild-type copy of *SEC24* on a plasmid that contains a *URA3* selectable marker, which allows for counterselection of the plasmid on the drug 5-fluororotic acid (5-FOA), a toxic intermediate in the uracil synthesis pathway (18). Cells that retain wild-type *SEC24* (and with it the *URA3* gene) are unable to grow in the presence of 5-FOA, whereas cells that can survive without wild-type *SEC24* remain viable. We transformed this strain with a library of multicopy plasmids that contain random genomic fragments and replica plated ∼5000 transformants onto media containing 5-FOA, yielding a small number of viable colonies. We rescued the plasmids from these colonies, retransformed them into the parental B-site mutant strain to confirm plasmid-dependent rescue and sequenced the ends of the inserts to determine the genomic region responsible for rescue (Figure 1A; Table 1). Each rescued clone contained readily identifiable candidate suppressors: four clones contained *BET1*, three clones contained *SMY2*, recently reported to rescue a temperature-sensitive allele of *SEC24* (19), and one clone contained *SEC22*. Surprisingly, no plasmids were identified that contained *SEC24* itself, suggesting that the screen was not saturated, or that the library was under-represented for *SEC24*. Individual candidate genes were subcloned from the genomic construct and confirmed as suppressors (Figure 1B).

Table 1: Suppressors of the *sec24*Δ + *sec24(L616W)* B-site mutant

Library plasmid	Genes present
pYAK001	[*PDR8*], *BOP2*, ***SEC22***, *DCS1*
pYAK002	[*NAS2*], *YIL006W*, *EPS1*, ***BET1***, *DRE3*
pYAK003	[*NAS2*], *YIL006W*, *EPS1*, ***BET1***, *DRE3*
pYAK004	[*NPL4*], *SEC66*, ***SMY2***, *UMP1*, [*SWD3*]
pYAK005	*SEC66*, ***SMY2***, *UMP1*, [*SWD3*]
pYAK007	[*SEC66*], ***SMY2***, *UMP1*, *SWD3*, *ECM31*
pYAK008	*YIL006W*, *EPS1*, ***BET1***, *DRE3*
pYAK009	*YIL006W*, *EPS1*, ***BET1***, *DRE3*

Gene names in bold were confirmed as the suppressing genes by subcloning and retransformation. Brackets indicate an interrupted ORF.

The ability of *BET1* to complement the B-site mutant was not surprising, as this essential SNARE is clearly

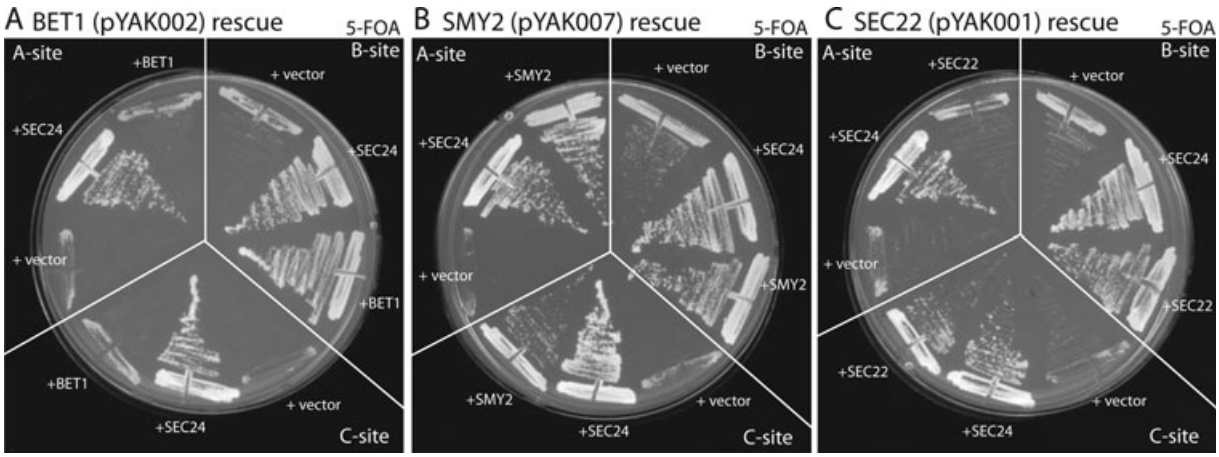

Figure 2: Cross-complementation of suppressing plasmids. The ability of suppressing plasmids to rescue multiple cargo-binding mutant alleles of *SEC24* was tested by expressing the rescuing plasmids in strains expressing A-site (W897A), B-site (L616W) and C-site (R342A) mutants as described in Figure 1. A) *BET1* overexpression was specific for the B-site mutant. B) *SMY2* was able to rescue all cargo-binding mutants. C) *SEC22* rescued both the B- and C-site mutants.

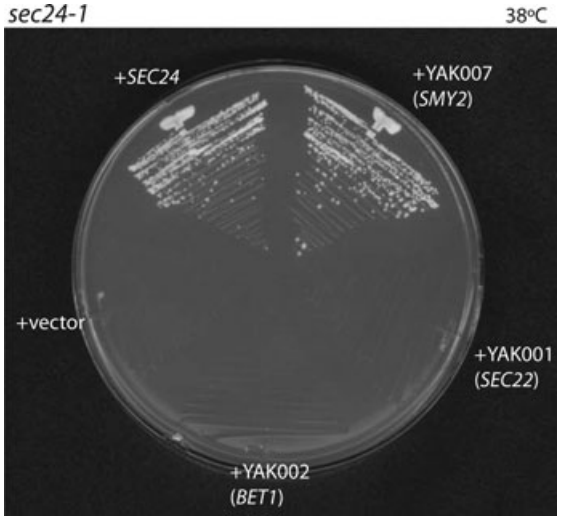

Figure 3: *SMY2* rescues the temperature-sensitive mutant, *sec24-1*. The temperature-sensitive strain, *sec24-1*, was transformed with the indicated plasmids and growth tested at 38°C: only *SMY2* overexpression was able to complement this mutation.

depleted in vesicles made with the L616W mutant of Sec24p (13). Indeed, testing cross-complementation of the three well-defined yeast cargo-binding mutants showed striking specificity of *BET1* rescue for the B-site mutant (Figure 2A). Conversely, overexpression of *SMY2* (Suppressor of *myo2*) rescued all three cargo-binding mutants (Figure 2B) as well as the temperature-sensitive *sec24-1* allele (Figure 3). *SMY2* suppression of the C-site mutant was slightly weaker than that observed for the other mutants but still represented significant rescue. This relatively broad suppression is consistent with a recent report that identified this gene as a suppressor of *sec24-20*, which contains a premature stop codon within the A-site (19). Given the general ability of *SMY2* to rescue multiple alleles of *SEC24*, as well as biochemical data showing that Smy2p is not packaged into COPII vesicles (19), we imagine that *SMY2* rescue is not linked to a function as an essential cargo protein but through a more general mechanism of enhancing secretion. Smy2p binds to the aberrant form of Sec24p (19), and differences in the degree of rescue of the three cargo-binding mutants may reflect distinct affinities for these altered proteins. *SEC22* likely represented a somewhat more specific suppressor, as it did not complement an A-site mutation (Figure 2C) nor a *sec24-1* allele (Figure 3). However, given that Sec22p is known to interact directly with the C-site of Sec24p, we were somewhat surprised to isolate it as a suppressor of the B-site mutant (Figure 2C).

The ability of *SEC22* to suppress the B-site mutant potentially represented a direct effect on cargo capture. We first explored the trivial possibility that Sec22p overexpression directly compensated for the Bet1p export deficit produced by the B-site mutation, in essence replacing the function of Bet1p in ER-to-Golgi transport. In this model, the B-site mutation could be functionally mimicking a loss-of-function Bet1p mutation by reducing the flux of this important SNARE through the early secretory pathway. We therefore tested whether *SEC22* overexpression could directly rescue a defect in Bet1p function by complementing a temperature-sensitive *bet1-1* allele. We introduced multicopy plasmids bearing either *BET1* or *SEC22* into the temperature-sensitive strains *bet1-1* and *sec22-3* and shifted cells to restrictive temperature (Figure 4). As expected, Bet1p overexpression rescued *bet1-1* and Sec22p overexpression rescued *sec22-3*; Bet1p overexpression also moderately rescued the *sec22-3* allele, as described earlier (20). However, Sec22p overexpression failed to rescue the *bet1-1* allele, which contains a substitution in the SNARE domain (21), suggesting that overexpressed Sec22p was not simply replacing the fusogenic function of Bet1p. Furthermore, overexpression of *SEC22* did not result in the upregulation of either Bet1p or Sed5p, as detected by immunoblotting (data not shown), suggesting that gene expression changes are not the proximal cause of rescue.

Cross-talk between the B- and C-sites in cargo capture

Sec22p has been well described as a C-site cargo protein by genetic, biochemical and structural studies (13,14). However, the uptake of Sec22p into COPII vesicles is also reduced when vesicles are made with the B-site Sec24p mutants. The B-site pocket and the surface encompassing the C-site on Sec24p are separated by a single loop, raising the possibility that B-site mutants may structurally perturb the C-site pocket (12,13). Thus, rescue of the B-site mutant by Sec22p could occur either by rescuing

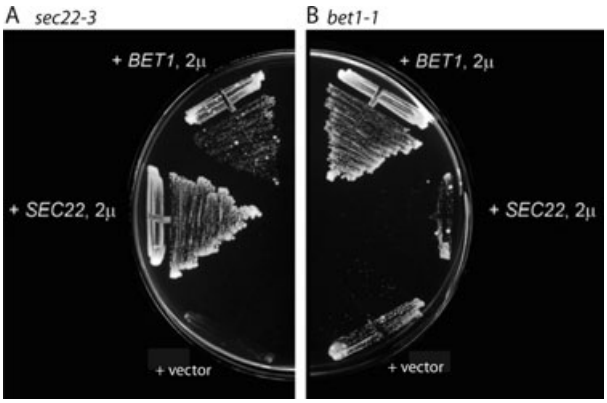

Figure 4: *SEC22* cannot rescue a defective *bet1* temperature-sensitive allele. The temperature-sensitive mutants *sec22-3* and *bet1-1* were transformed with multicopy vectors expressing either *BET1* or *SEC22* as indicated and growth was monitored at 37°C. A) Both *BET1* and *SEC22* overexpression were able to rescue the *sec22-3* allele. B) Conversely, overexpression of *SEC22* failed to rescue the *bet1-1* allele, which was rescued by *BET1* expression.

a Sec22p-specific budding defect associated with this mutation, or through an allosteric correction of the B-site defect by engagement of Sec22p with the C-site, or more indirectly through the enhanced packaging of Bet1p bound to Sec22p at the C-site. To more carefully explore these possibilities, we generated new *SEC24* alleles that we predicted would differentially affect either the B- or C-site. We mutated a pair of residues found on the loop separating the B- and C-sites (Figure 5A) and identified specific *in vitro* export deficits associated with the two mutations: the *sec24-R299A* allele created a true B-site defect, where Bet1p alone was reduced in its ER export; conversely, the *sec24-Q300R* mutation represents a C-site allele that is specifically deficient in Sec22p export (Figure 5B).

We took advantage of these specific export defects to discern whether *SEC22* overexpression rescues the L616W B-site mutation by compensating for a Sec22p deficit. Both the Q300R and R299A mutants were unable to confer viability in a *sec24Δiss1Δ* strain, allowing us to examine the ability of multicopy plasmids bearing either *BET1* or *SEC22* to rescue these mutants (Figure 5C). Sec22p overexpression rescued the *sec24-Q300R* C-site allele whereas Bet1p overexpression did not, as might have been expected given the lack of a Bet1p export deficit. However, both Bet1p and Sec22p were able to rescue the *sec24-R299A* B-site mutant, despite the lack of a Sec22p export defect for this mutant (Figure 5C). This suggests that Sec22p overexpression does not simply compensate for its own export defect in the B-site mutants and instead raises the possibility that increasing the concentration of Sec22p at the Sec24p C-site allosterically stabilizes the mutated B-site, thereby enhancing the export of a B-site cargo such as Bet1p.

Sec22p domain requirements for B-site rescue

To further dissect the mechanism by which Sec22p suppresses defects associated with mutation in the B-site, we explored the domain requirements for Sec22p rescue. We specifically aimed to examine potential cross-talk between the B- and C-sites by determining whether Sec22p functioned simply as a cargo protein or if its

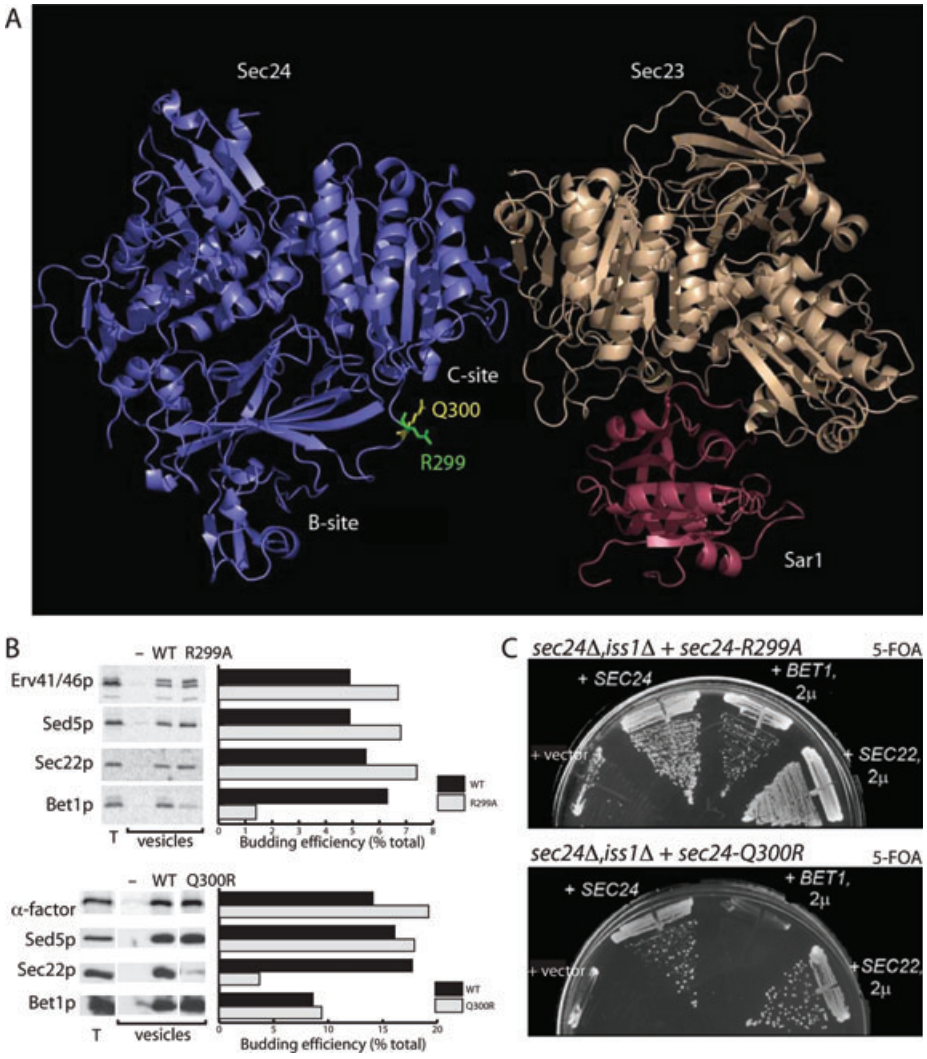

Figure 5: Novel *SEC24* mutants at the interface of the B- and C-sites differentially affect Bet1p and Sec22p export. A) A loop separating the B- and C-sites on Sec24p was targeted for mutagenesis, identifying two key residues, R299 and Q300, that fail to confer viability in a sec24Δiss1Δ double mutant. B) COPII vesicles were generated *in vitro* using either the Q300R or R299A mutant form of Sec24p. Packaging of the cargo proteins indicated was monitored by immunoblotting and quantified using ^{35}S-conjugated secondary antibodies. We observed a Bet1p-specific defect associated with the R299 mutant (top panel) and a Sec22p-specific defect associated with Q300 mutation (bottom panel). C) The ability of the R299A and Q300R mutants to be rescued by *SEC22* overexpression was tested by transforming the indicated strains with plasmids expressing *BET1* or *SEC22* as indicated. *SEC22* overexpression was able to rescue both Q300R and R299A mutants, whereas *BET1* overexpression only rescued the R299A mutant.

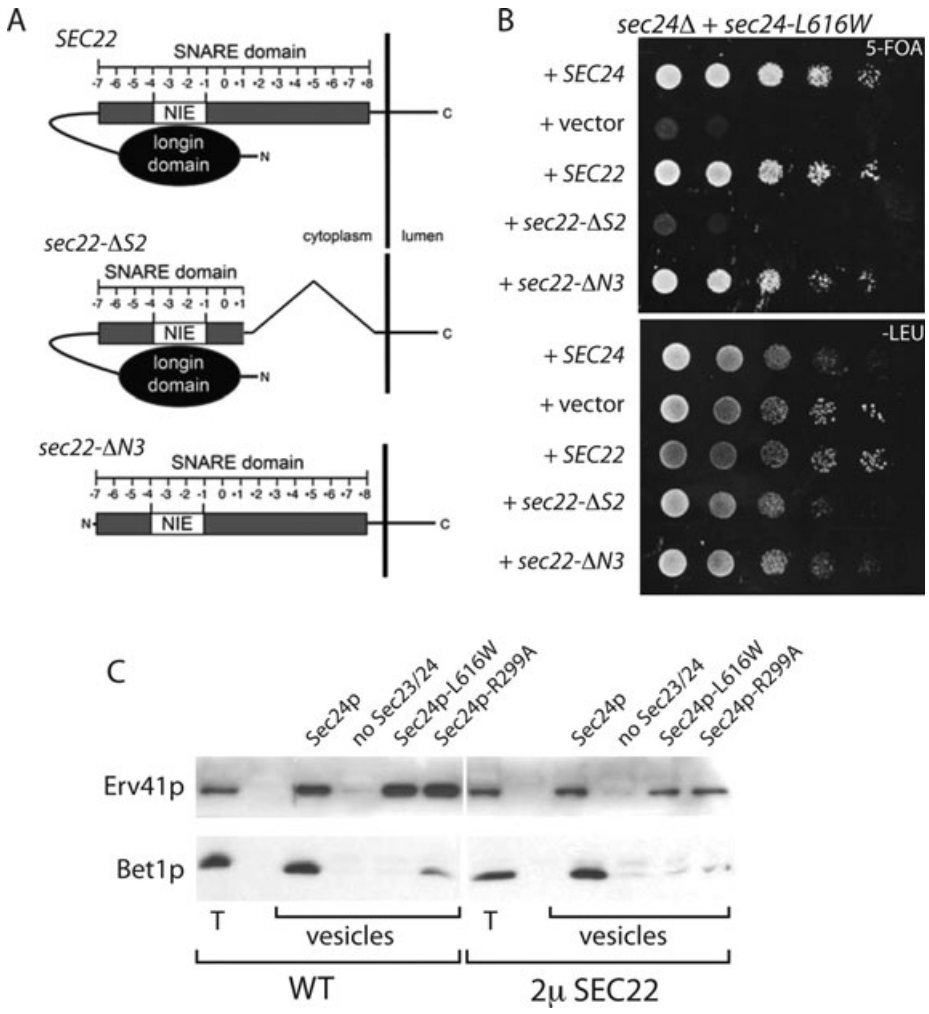

Figure 6: Sec22p mutants distinguish between a requirement for Sec24p binding and the Sec22p SNARE domain in rescue of Sec24p B-site mutants. A) Domain arrangement of Sec22p: the uptake into COPII vesicles is mediated by a folded epitope comprising the longin domain bound to an NIE motif within the SNARE domain. Numbering on the SNARE domain refers to the distance from the '0 layer'. B) The truncation mutants of Sec22p indicated in Figure 6A were tested for their ability to complement the B-site mutant of *SEC24*. Multicopy overexpression of wild-type (WT) *SEC22* robustly rescued the growth on 5-FOA of the *sec24-L616W* mutant, as did a *sec22-DN3* allele containing only the SNARE and transmembrane (TM) domains. The SNARE mutant *sec22-ΔS2* did not rescue. C) Microsomal membranes purified from WT cells (left panel) or a strain overexpressing *SEC22* (right panel) were used in COPII vesicle budding assays supplemented with the Sec24p mutants indicated. Vesicles were separated from total membranes (T) and packaging of Erv41p and Bet1p monitored by immunoblotting. Bet1p capture into vesicles in the presence of the L616W and R299A mutants was not rescued by overexpression of Sec22p.

action as a SNARE was required. The cytosolic region of Sec22p comprises an N-terminal longin domain of approximately 125 amino acids, followed by an ~60 residue SNARE domain that drives interaction with other SNAREs and subsequent membrane fusion (Figure 6A). Biochemical and structural experiments have indicated that the N-terminal longin domain plus a short segment of the SNARE domain are required for Sec22p to bind the interface between Sec23p and Sec24p (14,22). A truncated form of Sec22p lacking most of the C-terminal half of its SNARE domain, *sec22-ΔS2*, is deficient in binding its cognate ER-to-Golgi SNARE proteins Bet1p, Bos1p and Sed5p, but is still captured into COPII vesicles *in vitro* as it retains the conformational ER export epitope (22). If Sec22p rescues the B-site simply by binding the C-site and thereby stabilizing the B-site, *sec22-ΔS2* should suffice to rescue the Sec24p B-site mutants. We expressed *sec22-ΔS2* from a multicopy plasmid in the *sec24Δ + sec24-L616W* strain and tested for complementation on 5-FOA; *sec22-ΔS2* was unable to confer viability in this strain, indicating a requirement for an intact SNARE domain and suggesting that allosteric stabilization of the B-site is unlikely to be the mechanism of Sec22p-mediated rescue (Figure 6B). In further support of the essential function of the SNARE domain in rescue, a sec22-3 allele containing a mutation in the 0 layer was similarly unable to rescue the B-site (data not shown). Another possibility is that Sec22p rescues the B-site mutation by forming a SNARE complex with Bet1p, thus indirectly recruiting it into a COPII vesicle and bypassing the need for an intact B-site. We tested for this directly by examining Bet1p capture into COPII vesicles in the presence of Sec24p-L616W or Sec24p-R299A, and observed no detectable Bet1p in the vesicle fraction released from membranes overexpressing Sec22p (Figure 6C). As the *in vitro* budding reaction may not be sensitive enough to detect very small increases in Bet1p capture, we sought to confirm this

in vivo. Therefore, we tested suppression by a second Sec22p truncation, *sec22-ΔN3*, which lacks the N-terminal longin domain but retains the entire SNARE domain. As this truncation removes the conformational epitope recognized by Sec24p, it should no longer be recruited into COPII vesicles and remain ER retained. Indeed, similar N-terminal truncations of Sec22p largely retained their ability to interact with the cognate ER-to-Golgi SNAREs but were excluded from COPII vesicles (22). Overexpression of *sec22-ΔN3* was able to rescue the *sec24-L616W* B-site mutant, suggesting that the Sec22p SNARE domain alone is sufficient for rescue and that the uptake into COPII vesicles is not required for this *in vivo* complementation.

Discussion

Efficient egress of secretory proteins from the ER is fundamental to the viability of eukaryotic cells (23). In many cases, this process is mediated by a direct interaction between an ER export motif on a cargo protein and the Sec24p component of the COPII coat. Four distinct binding sites have been characterized biochemically, structurally and genetically in yeast and/or mammalian Sec24p. However, despite the wealth of mechanistic information about cargo selection by this protein, there remain many questions regarding the breadth of cargo recognition, the hierarchy of cargo capture and whether cargo binding by Sec24p causes allosteric changes that in turn influence additional cargo recruitment. We set out to use a genetic approach to further investigate cargo selection at the so-called B-site of Sec24p, which binds to a variety of related ER export signals.

Mutation of the Sec24p B-site produces *in vitro* export defects in multiple cargo proteins, including the essential SNAREs, Bet1p, Sed5p and Bos1p, the non-essential SNARE, Sec22p, and a variety of other transmembrane proteins (13). The peptide motifs that Bet1p, Sys1p and Sed5p use to engage this site have been defined biochemically and structurally, and interact with Sec24p via distinct binding modes despite engaging the same site (12). This suggests that the B-site is a multivalent export site capable of concentrating a diversity of cargo proteins. Given the essential nature of several of the cargoes engaged by the B-site, it is not surprising that mutation of this domain impairs viability when the mutant form is present as the sole copy of Sec24p. We sought to exploit this *in vivo* lethality in a multicopy suppressor screen to identify proteins that rescue viability in this strain background, and successfully isolated three independent suppressing genes, *BET1, SMY2* and *SEC22*, which seem to function via distinct mechanisms to improve the viability of the B-site mutant strain.

Bet1p is the vesicle-borne SNARE that is required on ER-derived vesicles in order for fusion at the Golgi to occur. *BET1* suppression of the B-site mutant likely directly rescues a functional Bet1p deficit, either by increasing the bulk flow transport of Bet1p out of the ER or by mass action driving increased binding to the crippled B-site. Furthermore, the isolation of *BET1* as a specific suppressor of the B-site mutant shows that this genetic approach has the capacity to identify *bona fide* cargo proteins. Indeed, we went on to show that Sec22p also rescues its site-specific mutation in Sec24p. Conversely, similar experiments with Sed5p and the A-site did not show suppression (data not shown), suggesting a more complicated repertoire of cargo proteins is responsible for the lethal phenotype of A-site mutants. The mechanism of suppression by *SMY2* is less clear: Smy2p is a peripheral membrane protein that localizes to the ER and appears to be a general suppressor of impaired ER-Golgi traffic. We found that *SMY2* overexpression not only suppressed the Sec24p B-site mutant but also rescued the A- and C-site mutants as well as the more generally defective *sec24-1* allele. Our findings are consistent with a recent report that identified *SMY2* as a suppressor of an additional Sec24p mutant, *sec24-20*, as well as other temperature-sensitive alleles in the early secretory pathway, including *sec16-2, sec22-3*, *bet1-1*, *sec34-1* and *sec35-1* (19). Smy2p itself was not found in ER-derived transport vesicles and was found in association with Sec23/24p only in the *sec24-20* mutant strain (19), suggesting it is not a *bona fide* cargo protein. Together, these data are consistent with a function for Smy2p as a general accessory protein that promotes flux of protein in the early secretory pathway, either by upregulating vesicle production at the ER or by promoting vesicle consumption at the Golgi, thereby overcoming both cargo-specific defects associated with mutation of Sec24p and more general deficiencies in protein biogenesis.

In contrast to the relatively broad rescue by Smy2p, Sec22p was more restrictive in its ability to complement specific Sec24p defects: both B- and C-site mutants were rescued whereas the A-site mutant and the more general *sec24-1* allele were not. The ability of Sec22p to rescue the B-site mutation was somewhat surprising, as the interaction between Sec22p and Sec24p clearly involves the C-site. That said, the B- and C-sites are adjacent to each other and mutation of the B-site partially impairs the *in vitro* packaging of Sec22p into COPII vesicles (13). We considered the possibility that Sec22p overexpression was simply rescuing this reduction in ER export *in vivo*, as well as the potential for functionally replacing Bet1p as a fusogenic SNARE. However, Sec22p overexpression did not rescue a temperature-sensitive *bet1-1* allele that is defective in the SNARE domain, and a new mutation in Sec24p created a truly B-site specific lesion that was still rescued by Sec22p. Combined with our dissection of the domains of Sec22p required for rescue, we conclude that binding of Sec22p to Sec24p is not required for rescue, but that a functional SNARE domain is necessary and sufficient to complement the B-site mutation. In principle, this domain could function in anterograde and/or retrograde transport as Sec22p acts in both pathways. However, our observed rescue of both the B- and C-site Sec24p mutants with a construct that would not be competent for

ER export, *sec22-ΔN3,* suggests that the most important function of Sec22p in these cells is as a retrograde SNARE receiving transport vesicles from the Golgi. Consistent with this model, N-terminal truncations in Sec22p that are not packaged into COPII vesicles are also capable of complementing a *sec22Δ* mutant (22). Although non-specific or bulk flow transport of truncated Sec22p may permit a limited amount of ER export that might complement a lack of Sec22p, such a low level of Sec22p flux is difficult to reconcile with a rescue of a Bet1p (or other essential cargo) defect. Therefore, we favor the hypothesis that increased abundance of Sec22p in the ER membrane promotes the efficient retrograde retrieval of the ER-Golgi SNAREs and thereby increases the local concentration of Bet1p to sufficient levels to overcome a reduction in packaging associated with the B-site mutation.

Our observed lack of cross-talk between cargo-binding sites on Sec24p is consistent with structural data that show no major rearrangements of cargo-bound relative to cargo-free forms of Sec24p (12,14,24). However, most of these structures were solved with small peptides occupying the cargo-binding sites, leaving the formal possibility that some structural changes might occur in the context of a larger cargo molecule. Furthermore, there remains the possibility that allostery does operate in promoting association of some specific cargoes, either at different sites on Sec24p or by specific mechanisms at the B- and C-sites that remained undetected in our analysis. However, of the known cargo-binding sites, the immediately adjacent B- and C-sites would seem prime candidates for regulation in this manner. In particular, one might imagine that binding of Bet1p, the vesicle-associated SNARE required for delivery to the Golgi, might stimulate subsequent recruitment of additional cargoes such that secretory proteins optimally engage Sec24p when it is already bound to this critical delivery component. Such cross-talk would ensure that cargo-containing vesicles are programmed for fusion by specifically expanding cargo selection around the SNARE-occupied site. A more expansive description of the full repertoire of cargo proteins that engage the known (and potentially novel additional) sites on Sec24p might aid in the identification of such regulation.

Materials and Methods

Yeast strains and media

Strains used in this study are listed in Table 2. With the exception of temperature-sensitive strains, yeast cultures were grown at 30°C in synthetic complete (SC) media (SC: 0.67% yeast nitrogen base, 2% carbon source, supplemented with amino acids as required). Yeast transformations were performed through standard lithium acetate yeast transformation methods (25). Temperature-sensitive strains were grown at 25°C and then shifted to 37°C, except for *sec24-1*, which was grown at 30°C to test complementation at restrictive temperature. Suppression analysis was performed by replica plating library transformants first onto 2% agar plates to reduce background growth and then onto SC supplemented with 5-FOA (0.1% of final concentration). Transformants that suppressed the lethal phenotype were reconfirmed by streaking or spotting serially diluted saturated overnight cultures onto SC supplemented with 5-FOA.

Plasmid construction

Plasmids used in this study are listed in Table 3. To construct *BET1*_425 and *SEC22*_425, 1.3-kb fragments containing the open reading frame (ORF) of the genes of interest and 5′- and 3′-untranslated regions (UTRs) extending up to the neighboring ORFs were first cloned into the *Eco*RI/*Xho*I sites in pBluescript II KS, resulting in *BET1*_pBS and *SEC22*_pBS, respectively. The inserts were then subcloned into the *Bam*HI/*Xho*I sites of pRS425 to produce the plasmids *BET1*_425 and *SEC22*_425. A 1.4-kb fragment containing the ORF of *SED5* and a 5′-UTR extending up to the neighboring ORF were cloned into the *Bam*HI/*Xho*I sites of pRS425 to create *SED5*_425. *SMY2*_425 was constructed by cloning a 2.6-kb fragment containing the *SMY2* gene and a 5′-UTR extending up to the neighboring ORF into the *Spe*I/*Xho*I sites of pRS425. The Sec22 SNARE domain partial deletion was constructed by restriction digesting *Sec22-SE-B* (22) with *Mfe*I and religating the plasmid, resulting in a 1.5-kb fragment consisting of the *SEC22* gene, including 5′- and 3′-UTRs, with a deleted segment from nucleotides 489 to 555 cloned into the *Bam*HI/*Eco*RV sites of pRS313, creating pRB058. The construct was subcloned into the *Spe*I/*Xho*I sites of pRS425 to produce pRB059. The N-terminally truncated Sec22 was produced by blunt end ligating the 5′-UTR of *SEC22*, including the endogenous start codon, to a segment beginning at nucleotide 388 through the end of *SEC22* and a portion of the 3′-UTR. The 1.3-kb fragment was cloned into the *Spe*I/*Xho*I sites of pBluescript II KS to create pRB080 and then subcloned into the *Spe*I/*Xho*I sites of pRS425 to produce pRB085. Plasmids pLM22, pLM23, pLM137, pLM171 and pLM25 were previously described (13,27). Point mutations in *SEC24* were introduced by site-directed mutagenesis of pLM23 to obtain pLM171 and pRB074.

In vitro *budding*

Microsomal membranes were purified from RSY620 cells as described previously (28). Membranes were washed with 2.5 M urea in B88 buffer

Table 2: Strains used in this study

Strain	Genotype	Source
LMY287	*MATα ade2-101cc his3Δ200 leu2Δ1 lys2-801am trp1Δ63 ura3-52 sec24::TRP1* carrying pLM22 *(CEN SEC24-URA3)*	(13)
RBY001	*MATα ade2-101cc his3Δ200 leu2Δ1 lys2-801am trp1Δ63 ura3-52 sec24::TRP1 iss1::KanR* carrying pLM22 *(CEN SEC24-URA3)*	This study
RBY008	LMY287 carrying pLM137	This study
RBY018	RBY001 carrying pLM134	This study
RBY025	RBY001 carrying pLM174	This study
RBY026	RBY001 carrying pLM251	This study
Sec24-1	*MATα leu2,3-112 ura3-52 sec24-1*	Schekman strain collection
Bet1-1	*MATα his4-619 ura3-52 bet1-1*	(20)
Sec22-3	*MATα SUC2 mal0 gal2 CUP1 ura3-52 sec22-3*	(26)
RSY620	*MATα leu2-3,112 ura3-52 ade2-1 trp1-1 his3-11,15 pep4::TRP1*	Schekman strain collection

Table 3: Plasmids used in this study

Plasmid	Description	Source
pLM22	4.2-kb *Xho*I-*Spe*I fragment containing *SEC24* in pRS316 (*URA3*, CEN)	(13)
pLM23	4.2-kb *Xho*I-*Spe*I fragment containing *SEC24* in pRS313 (*HIS3*, CEN)	(13)
pLM134	Sec24R230,235A mutation in pLM23	(13)
pLM137	Sec24L616W mutation in pLM23	(13)
pLM174	Sec24R342A mutation in pLM23	(13)
pLM251	Sec24W897A mutation in pLM23	(27)
pLM171	Sec24R299A mutation in pLM23	This study
pRB074	Sec24Q300R mutation in pLM23	This study
*BET1*_pBS	1.3-kb *Eco*RI-*Xho*I fragment containing *BET1* in pBluescript II KS	This study
*BET1*_425	1.3-kb *Bam*HI-*Xho*I fragment containing *BET1* in pRS425 (*LEU2*, 2μ)	This study
*BET1*_426	1.3-kb *Bam*HI-*Xho*I fragment containing *BET1* in pRS426 (*URA3*, 2μ)	This study
*SEC22*_pBS	1.3-kb *Eco*RI-*Xho*I fragment containing *SEC22* in pBluescript II KS	This study
*SEC22*_425	1.3-kb *Bam*HI-*Xho*I fragment containing *SEC22* in pRS425 (*LEU2*, 2μ)	This study
*SEC22*_426	1.3-kb *Bam*HI-*Xho*I fragment containing *SEC22* in pRS426 (*URA3*, 2μ)	This study
*SED5*_425	1.4-kb *Bam*HI-*Xho*I fragment containing *SED5* in pRS425 (*LEU2*, 2μ)	This study
AB320 library	Genomic fragments cloned in *Bam*HI cut Yep13 (*LEU2*, 2μ)	ATCC
*SMY2*_425	2.6-kb *Spe*I-*Xho*I fragment containing *SMY2* in pRS425 (*LEU2*, 2μ)	This study
Sec22-SE-B	1.5-kb *Bam*HI-*Eco*RI fragment containing *sec22(+489 MfeI, +555 MfeI)* in pRS313 (*HIS3*, CEN)	(22)
pRB059	1.5-kb *Spe*I-*Xho*I fragment containing *sec22(DS2)* in pRS425 (*LEU2*, 2μ)	This study
pRB080	0.9-kb *Spe*I-*Xho*I fragment containing *sec22(DN3)* in pBluescript II KS	This study
pRB085	0.9-kb *Spe*I-*Xho*I fragment containing *sec22(DN3)* in pRS425 (*LEU2*, 2μ)	This study

(20 mM HEPES, pH 7.4, 250 mM sorbitol, 160 mM potassium acetate, 5 mM magnesium acetate) and twice with B88 buffer without urea. Purified wild-type Sec24 or mutant Sec24 and other COPII proteins (10 μg/mL Sar1p, 10 μg/mL Sec23p/24p and 20 μg/mL Sec13/31p) were incubated with 125 μg of membranes per reaction either in the presence of 0.1 mM GTP with a 10× ATP regeneration system or 0.1 mM GDP. The vesicles were separated from donor membranes by centrifugation at 16 000 × ***g*** for 5 min and the vesicle-containing supernatant was further concentrated by high-speed centrifugation at 112 000 × ***g*** for 20 min. Vesicle pellets were resuspended in SDS sample buffer and heated at 55°C for 5 min before separation by SDS–PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and analyzed by quantitative immunoblotting using ^{35}S-conjugated anti-rabbit immunoglobulin G (IgG). α-Factor, Sed5p, Sec22p, Bet1p and Erv41p/46p were detected with polyclonal antibodies, gifts from C. Barlowe (Dartmouth Medical School) and R. Schekman (UC Berkeley).

Protein purification

Sar1p, Sec13p/31p and wild-type Sec23p/24p were purified as previously described (16). Sec24p mutants were co-expressed with Sec23p in RSY620 cells under the control of the *GAL1* inducible promoter. Cells were grown in SC with raffinose as the carbon source and induced with 0.2% galactose. Mutant Sec24 protein was co-purified with endogenous Sec23p as described for the wild-type Sec23p/24p complex.

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