Multiple Cargo Binding Sites on the COPII Subunit Sec24p Ensure Capture of Diverse Membrane Proteins into Transport Vesicles

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Summary

We have characterized the mechanisms of cargo selection into ER-derived vesicles by the COPII subunit Sec24p. We identified a site on Sec24p that recognizes the v-SNARE Bet1p and show that packaging of a number of cargo molecules is disrupted when mutations are introduced at this site. Surprisingly, cargo proteins affected by these mutations did not share a single common sorting signal, nor were proteins sharing a putative class of signal affected to the same degree. We show that the same site is conserved as a cargo-interaction domain on the Sec24p homolog Lst1p, which only packages a subset of the cargoes recognized by Sec24p. Finally, we identified an additional mutation that defines another cargo binding domain on Sec24p, which specifically interacts with the SNARE Sec22p. Together, our data support a model whereby Sec24p proteins contain multiple independent cargo binding domains that allow for recognition of a diverse set of sorting signals.

Introduction

Transport through the secretory pathway is mediated by vectorial transfer of proteins between compartments within membrane bound vesicles. Generation of vesicles is achieved by compartment-specific cytoplasmic coat proteins that function both in membrane deformation and compartmental organization such that cargo molecules are included in nascent vesicles, while residents are excluded. This process of cargo selection is thought to be driven by direct interaction of coat proteins with specific sorting signals that are responsible for directing uptake of cargo molecules into a budding vesicle (Aridor and Traub, 2002; Schekman and Orci, 1996). Different cytoplasmic coat proteins mediate transport between the various compartments of the secretory pathway, each recognizing distinct sorting signals borne by cargo molecules.

The COPII coat, composed of five proteins that comprise three subunits (Sec13, Sec23, and Sec31), generates vesicles from the endoplasmic reticulum (ER) that are destined to fuse with the Golgi apparatus, the first vesicular step in forward transport of secretory proteins (Barlowe et al., 1994). The process of cargo selection by the COPII coat is thought to be facilitated by the Sec24p subunit. Sec23/24p binds to cargo molecules in vitro (Peng et al., 1999; Springer and Schekman, 1998; Votsmeier and Gallwitz, 2001), and cargo-containing “prebudding” complexes can be isolated after incubation of ER membranes with Sar1p and Sec23/24p (Aridor et al., 1998; Kuehn et al., 1998). Furthermore, a homolog of Sec24p, Lst1p, is specifically required for efficient packaging of the plasma membrane protein Pma1p (Roberg et al., 1999; Shimoni et al., 2000). Moreover, vesicles generated with Sec23/Lst1p in the absence of Sec24p contain a unique subset of cargo molecules, suggesting distinct cargo specificities for Sec24p and Lst1p (Miller et al., 2002).

The sorting signals that govern uptake into COPII vesicles are relatively poorly defined. In contrast to the well-characterized signals that mediate endocytosis and retrograde transport between the Golgi and ER, there is no apparent universal ER export signal that is present on all secretory proteins. Recent work characterizing the cytoplasmic domains of a variety of cargo molecules from diverse systems has revealed two classes of ER export signals. A dihydrophobic motif, often consisting of two C-terminal hydrophobic residues, facilitates export of the p24 family of proteins (Nakamura et al., 1998) as well as the ERGIC53/Emp47 proteins (Kappeler et al., 1997; Sato and Nakano, 2002). A second class of signal, a diacidic motif, directs ER export of the viral, mammalian, and yeast proteins, VSV-G, Kir1.1/Kir1.2, and Sys1p, respectively (Nishimura and Balch, 1997; Stockklausner et al., 2001; Votsmeier and Gallwitz, 2001). However, in the case of VSV-G, additional residues also contribute to efficient packaging (Nishimura et al., 1999; Sevier et al., 2000). Many of these ER export signals have been shown to bind to the COPII subunit Sec23/24p. Importantly, mutation of the sorting motif abrogates both Sec23/24p binding and ER export (Aridor et al., 2001; Belden and Barlowe, 2001a; Nufer et al., 2002).

Here, we describe the characterization of the molecular mechanisms of cargo recruitment by the Sec24p subunit of the COPII coat. Using an alanine-scanning mutagenesis approach, we have identified and characterized mutations in Sec24p that specifically disrupt recruitment of a subset of cargo molecules. Our data suggest that Sec24p has multiple independent sites of cargo recognition. Together with a detailed structural and biochemical analysis of coat-SNARE interactions (Mossessova et al., 2003), these functional studies demonstrate that cargo recruitment into nascent vesicles is driven by direct interaction between a sorting signal and a coat subunit and provide an important basis for identifying distinct sorting signals and novel cargo interaction domains.

Results

The Sec23/24p complex of the COPII coat binds avidly to the cytoplasmic domains of a number of cargo mole-
the wild-type Sys1 peptide (Figure 1A). However, a peptide that contained mutations in the residues that make critical contact with Sec23/24p (D198AxE200A) failed to compete with GST-Bet1p for interaction with Sec23/24p (Figures 1A and 1C). Having demonstrated a direct competition between Bet1p and Sys1p for interaction with Sec23/24p, we investigated whether an additional cargo molecule with a distinct sorting motif could compete for the same site. The C-terminal cytoplasmic domain of Prm8p contains a diphenylalanine motif that is required for interaction with Sec23/24p (S. Springer and R.S., unpublished data). A peptide corresponding to this region binds avidly to Sec23/24p (S. Springer and R.S., unpublished data) and was tested for its ability to compete with GST-Bet1p. Unlike the Sys1p signal, neither the wild-type Prm8p signal, nor a mutant form that lacked the diphenylalanine motif was able to compete with GST-Bet1p for Sec23/24p binding (Figures 1B and 1C). These data suggest that Sys1p and Bet1p bind to the same site on Sec24p, which is likely to have another independent binding site that may interact with additional cargo molecules.

Cargo Binding Mutations in Sec24p

We undertook a genetic approach to identify functionally important residues on Sec24p that might be involved in cargo selection. The recent structure of the Sec23/Sec24/Sar1p complex (Bi et al., 2002) allowed the use of targeted alanine-scanning mutagenesis to alter charged residues around the equatorial (i.e., neither membrane proximal nor membrane distal) surface of Sec24p. One of these mutants, Sec24R230,235A, has alterations in a highly conserved region of Sec24p that was identified as the Bet1p/Sys1p binding site through X-ray crystallography (Mossessova et al., 2003 [this issue of Cell]). Using this structural information, we made several additional mutations in residues that make contact with Bet1p/Sys1p that would be expected to disrupt interaction with cargo molecules at this site. The two Arg residues identified in the original isolate were mutated individually (Sec24R230A and Sec24R559M), an additional two Arg residues were changed to Met (Sec24R230A and Sec24R559M), and a Leu residue that forms the base of the Sys1p binding pocket was mutated to Trp (Sec24L616M). The introduction of a bulky side chain at this position was expected to sterically interfere with the interaction between Sec24p and sorting signals and therefore abolish binding.

Hexahistidine-tagged versions of these mutant proteins were co-overexpressed with Sec23p and the complexes purified by Ni²⁺-affinity chromatography. Two mutants, Sec24R230A and Sec24R559M, were unstable on overexpression in yeast and were therefore omitted from further biochemical analysis. We first tested the mutant proteins in the GST-Bet1p binding assay described above. As expected, none of the mutants was able to bind to GST-Bet1p, confirming the critical role for this domain in the Sec24-Bet1p interaction (Figure 2A). We next used the mutant proteins in in vitro budding reactions that generate vesicles during an incubation of purified COPII components with ER-enriched micro-
Multiple Cargo Binding Domains on Sec24p

A GST-Bet1p was incubated with Sar1p (30 μg/ml) and wild-type or mutant versions of Sec23/24p (30 μg/ml) in the presence of guanine nucleotide (0.1 mM) as indicated. GSHA bound proteins as well as a fraction (10%) of the total reaction were resolved by SDS-PAGE and SYPRO staining. Only wild-type Sec23/24p was recruited to the GST-Bet1p beads.

(B) Total membranes (T) and budded vesicles were collected from in vitro budding reactions containing Sar1p (10 μg/ml), Sec13/31p (20 μg/ml), and nucleotide (0.1 mM), supplemented with wild-type or mutant Sec23/24p (10 μg/ml) as indicated. Proteins were detected by immunoblotting with antibodies against the proteins indicated.

(C) Packaging efficiency was determined by quantitative immunoblotting using radiolabeled secondary antibody. Packaging of each protein with wild-type Sec23/24p was set to 100% and the relative proportion of packaging with mutant Sec23/24p complexes was calculated.

(D) In vitro packaging of 35S-labeled α-factor into a vesicle fraction was determined by precipitation of glycosylated α-factor from the medium-speed supernatant after incubation with Sar1p (10 μg/ml), Sec13/31p (20 μg/ml), and wild-type or mutant Sec23/24p (10 μg/ml) and nucleotide as indicated.

(E) Vesicles generated as in (D) were incubated with acceptor Golgi membranes in the absence or presence of cytosol. Fusion of vesicles with the Golgi was determined by precipitation with antibodies against an α1,6 mannose moiety and scintillation counting.

We were able to demonstrate that the mutant Sec24p complexes were not broadly defective in generating COPII vesicles by examining packaging of the soluble cargo protein pro-α-factor. In a different in vitro budding reaction, microsomes containing radiolabeled pro-α-factor were washed with urea and incubated with wild-type or mutant proteins to generate COPII vesicles. Donor membranes were removed by medium-speed centrifugation, and the degree of glycosylated pro-α-factor packaging was quantified by Concanavalin A precipitation and scintillation counting (Barlowe et al., 1994). All mutants were able to package pro-α-factor into COPII vesicles to the same extent as wild-type Sec23/24p (Figure 2D). We next assessed the ability of the vesicles generated with mutant proteins to fuse with the Golgi apparatus by incubating vesicles with acceptor membranes and cytosol followed by immunoprecipitation of an α1,6-mannose moiety, a Golgi-specific modification (Barlowe et al., 1994). Vesicles generated with wild-type Sec23/24p were capable of fusion in a cytosol-dependent manner, whereas vesicles generated with any of the Sec24p mutants were impaired in their ability to fuse, consistent with the absence of SNAREs in these vesicles (Figure 2E). These data demonstrate a direct correlation between cargo binding by Sec24p and the corresponding presence or absence of that cargo molecule in COPII vesicles, reinforcing the hypothesis that it is the direct interaction between a Sec24p subunit and a sorting motif that drives cargo recruitment into COPII vesicles.

We sought to characterize the larger role of this cargo binding domain of Sec24p in protein sorting. To this end, we surveyed a number of cargo molecules for their...
Figure 3. Examination of the Effect of Sec24L616W on Packaging of a Range of Cargo

Vesicles were generated from microsomal membranes using either Sec23/24p or Sec23/24L616W, as described in Figure 2B. The presence of a number of cargo molecules was determined by immunoblotting or by autoradiography (35S-labeled α-factor) and quantified using radiolabeled secondary antibody. The ER export signals that direct forward transport are indicated. In this figure, footnote abbreviations for signal types refer to specific references: a, Malkus et al. (2002); b, Otte and Barlowe (2002); c, Sato and Nakano (2002); d, Mossessova et al. (2003); e, Nakamura et al. (1998); and f, Votsmeier and Gallwitz (2001) and this study.

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This cargo binding domain (data not shown). Interestingly, the packaging efficiency of these unaffected molecules was often greater in the presence of mutant Sec23/24p than with wild-type Sec23/24p. Cargo molecules that were impaired in their uptake into COPII vesicles in the presence of the mutant Sec23/24p complex fell broadly into three classes: those that were moderately affected (∼50% relative to wild-type), moderately affected (∼20% of wild-type), or severely affected (<10% of wild-type). Moderately affected proteins included the Golgi t-SNARE Sed5p, which employs a bipartite sorting signal, of which one motif resembles the Bet1p sorting signal (Mossessova et al., 2003). Also in this class was Emp47p, which contains a tyrosine motif that is important for ER export and COPII binding. The mammalian homolog of Emp47p, ERGIC53, also contains a dihydrophobic motif that is required for its correct localization (Nufer et al., 2002). Intermediately affected proteins included the SNAREs described above, Bos1p and Sec22p, which contain uncharacterized sorting signals. Also among the intermediately affected proteins were the p24 proteins Emp24p, Erp1p, and Erp2p (Figure 3 and data not shown). These proteins all share a pair of hydrophobic residues at their extreme...
C termini that appear to be involved in ER export (Nakamura et al., 1998). Finally, the most severely affected proteins included Bet1p and a Gap1p/Sys1p chimera, which depends entirely on the Sys1p diacidic motif for uptake into COPII vesicles (see Figure 5 below). Both of these proteins are likely to bind directly to the site targeted for mutagenesis (Mossessova et al., 2003).

We next examined the morphology of vesicles generated during the in vitro budding reaction by thin-section electron microscopy. The coat morphology on vesicles generated with Sec23/24L616W was indistinguishable from that seen on wild-type vesicles (Figures 4A and 4B). However, statistical analysis showed that vesicles generated with mutant protein were measurably smaller than those made with wild-type Sec23/24p. Vesicles generated with wild-type Sec23/24p had a mean diameter of 80 ± 2 nm, whereas vesicles generated with Sec23/24L616W were only 74 ± 5 nm in diameter (values are mean ± SD; p < 0.001). One explanation for this observation is that the reduction in the number of cargo molecules packaged into vesicles generated with mutant protein causes a reduction in vesicle size. Alternatively, mutation of the Sys1p binding pocket may alter the architecture of the coat subtly to produce a more tightly curved membrane. The vesicle morphology data confirm that the mutant Sec23/24p complex is capable of generating COPII vesicles and raise the intriguing possibility that cargo abundance can influence the architecture of a vesicle.

Not All Diacidic Sorting Signals Are Equal

The surprising observation that packaging of a second protein containing a diacidic sorting signal, Gap1p, was unaffected by mutation in the Bet1p/Sys1p binding pocket led us to investigate the relationship between the Sys1p and Gap1p signals. Other than in the superficially similar sorting signals, there is little conservation between the two proteins in the overall sequence of their cytoplasmic tails and the position of the diacidic residues relative to the final transmembrane domain (Figure 5A). We examined the in vitro packaging efficiency of truncated and tail-swapped chimerae of Gap1p and Sys1p. Using radiolabeled semi-intact cells as a source of donor membranes for the in vitro budding reaction, packaging of newly synthesized protein was assessed with wild-type COPII proteins (Figure 5B). As previously demonstrated (Malkus et al., 2002), full-length Gap1p was packaged into vesicles (~19%) and removal of the C-terminal domain dramatically reduced the packaging efficiency (~2%). Appending the Sys1p C-terminal domain to truncated Gap1p stimulated the packaging efficiency 5-fold to approximately 10%. Like Gap1p, Sys1p was efficiently packaged into COPII vesicles (~25%); however, removal of the Sys1p C-terminal domain had
Figure 5. The Diacidic Signals of Gap1p and Sys1p Are Distinct

(A) The C-terminal domains of Gap1p and Sys1p were compared using ALIGN. Gray boxes indicate diacidic sequences that are involved in ER export.

(B) Chimerae representing the first 535 residues of Gap1p or 150 residues of Sys1p (N-terminal region) were fused to the C-terminal domains of Gap1p, Sys1p, or a truncated version of the Gap1p C-terminal domain (ΔCT). Radiolabeled semi-intact cells were prepared from cells expressing these chimerae and used in in vitro COPII budding assays containing Sar1p (20 μg/ml), Sec23/24p (20 μg/ml), and Sec13/31p (40 μg/ml). Packaging efficiency was determined by immunoprecipitation, SDS-PAGE, and autoradiography. The percentage of labeled chimera in the vesicle fraction is plotted as a percentage of total labeled protein.

(C) Vesicle budding assays were performed for the indicated chimerae using either wild-type Sec23/24p or Sec23/24L616W. Packaging efficiency was determined as for (B) and plotted as a normalized packaging efficiency such that packaging with wild-type Sec24p was set to 100%.

a less dramatic affect on packaging, reducing the efficiency only 2.5-fold to approximately 10%. Surprisingly, appending the Gap1p C-terminal domain to truncated Sys1p yielded no increase in packaging efficiency (~10%). Together, these observations indicate that the diacidic ER export signals in Sys1p and Gap1p are not interpreted in the same way by the budding machinery.

We next examined how mutations in Sec24p affect the packaging efficiency of newly synthesized Gap1p, Sys1p, and Gap1/Sys1p chimera (Figure 5C). As noted previously for steady-state Gap1p (Figure 3), packaging into COPII vesicles was unaffected by the Sec24L616W mutation. However, packaging of Sys1p was reduced to approximately 30% of wild-type. Strikingly, packaging of the Gap1/Sys1p chimera was even more dramatically affected by the Sec24L616W mutation: budding efficiency was reduced to approximately 2% of wild-type. These data suggest that when packaging is determined solely by the Sys1p C-terminal domain (as in the Gap1/Sys1p chimera), disruption of the interaction with Sec24p completely abolishes ER export. Furthermore, they demonstrate that although the diacidic motif in Sys1p is important for ER export, there is some degree of packaging that is independent of this motif and likely resides outside of the C-terminal domain.

Functional Redundancy of Sec24p Homologs

We next tested the effects of mutation of the Bet1p/Sys1p binding site in vivo. Plasmids bearing the mutant versions of Sec24p were transformed into a yeast strain that contained a chromosomal deletion of the SEC24 gene, covered by a copy of SEC24 on a plasmid containing the URA3 marker gene (see Table 1 for yeast strains and plasmids). Transformants were grown on plates containing 5-FOA to counterselect for the wild-type SEC24 gene. Whereas all of the Arg mutants were able to complement the loss of Sec24p, cells expressing Sec24L616W were unable to grow in the absence of wild-type Sec24p (Figure 6A, left). After counterselection on 5-FOA to cure cells of the wild-type Sec24p, we tested the phenotypes of the Arg mutants at various temperatures. Sec24R230A grew poorly at 30°C and was unable to grow at either 25°C or 37°C. Sec24R561M showed a similar phenotype, whereas both Sec24R235A and Sec24R559M were viable at a range of temperatures (data not shown). The steady-state abundance of each mutant protein was similar to wild-type, suggesting that the observed phenotypes were not caused by increased turnover of Sec24p (data not shown).

We sought to address whether either of the Sec24p homologs, Iss1p and Lst1p, were required for the viability of Sec24p mutant cells. We generated strains that contained chromosomal deletions of SEC24, ISS1, and/or LST1 and tested the ability of the mutant versions of Sec24p to complement loss of plasmid-borne SEC24. The pattern of complementation in a sec24Δlst1 strain
was identical to that of the .sec24 strain (data not shown). However, the temperature-sensitive mutants Sec24R230A, Sec24R230,235A, and Sec24R561M were inviable in a .sec24/iss1 strain, suggesting that Iss1p can partially compensate for these lesions in Sec24p (Figure 6A, middle). Furthermore, the remaining mutants, Sec24R235A and Sec24R559M, were unable to complement a .sec24/iss1/iss7 strain (Figure 6A, right), suggesting roles for both Iss1p and Lst1p in the viability of cells expressing these mutants. These data suggest that the Sec24p homologs Iss1p and Lst1p can cooperate to facilitate intracellular transport in vivo in the presence of mutant forms of Sec24p.

We have previously reported that vesicles generated with Lst1p alone contain the GPI-anchored protein Gas1p, which lacks a cytoplasmic domain (Miller et al., 2002). Because Gas1p transport requires Emp24p, which is presumed to act as a receptor (Muniz et al., 2000), Lst1p may package Gas1p indirectly through contact with Emp24p, which displays a cytoplasmic domain known to interact with Sec23/24p (Belden and Barlowe, 2000). Because Gas1p transport requires Emp24p, which is presumed to act as a receptor (Muniz et al., 2000), Lst1p may package Gas1p indirectly through contact with Emp24p, which displays a cytoplasmic domain known to interact with Sec23/24p (Belden and Barlowe, 2000). Given that several p24 proteins were markedly impaired in their uptake into vesicles generated with the Sec24p mutants, we sought to address whether the equivalent mutations in Lst1p would similarly affect packaging of p24 proteins. Two double mutations were made in Lst1p: Lst1R219,224A, which corresponds to our original Sec24R230,235A mutant, and Lst1K543M, R545M, analogous to the Sec24R559M and Sec24R561M mutations. Purified Sec23/Lst1p complexes were used in vitro budding reactions and capture of a number of cargo molecules examined (Figure 6C). In addition to the p24 proteins Erp1p, and Emp24p, wild-type Lst1p was also capable of packaging Erp46p and pro-α-factor, albeit less efficiently than Sec24p. However, wild-type Lst1p was unable to package the amino acid permease Hip1p, nor the SNAREs Bet1p, Bos1p, or Sed5p (Figure 6C and data not shown). Like the equivalent Sec24p mutants, the Lst1p mutants were unable to incorporate either Erp1p or Emp24p into vesicles, whereas Erp46p and pro-α-factor were packaged relatively well. Quantl-
Figure 6. Functional Complementation of Sec24p Mutants by Iss1p and Lst1p via a Common Site of Cargo Interaction

(A) The ability of sec24 mutants to complement the loss of SEC24 was tested by growing RSY875 transformed with plasmids as indicated and growing cells on 5-fluoroorotic acid (5-FOA) to cure cells of the wild-type SEC24. Sec24L616W was unable to complement deletion of SEC24, and all other mutants were viable. Mutants were also tested for viability in cells that contained chromosomal deletions in ISS1 and LST1 in combination with SEC24, revealing additional phenotypes.

(B) Structural domain representation of Sec24p, highlighting the degree of conservation among Sec24p homologs at the Bet1p/Sys1p site and indicating the specific mutations (closed circles) that show defects in cargo packaging.

(C) In vitro budding reactions show that Lst1p packages the p24 proteins Emp24p and Erp1p, as well as Erv46p and pro-α-factor, whereas the Lst1p mutants are specifically unable to package the p24 proteins. Some proteins, including the SNAREs, Bet1p, Sed5p, and the amino acid permease Hip1p, are not packaged by any of the Lst1p proteins.

Identification of a Second Cargo Binding Site on Sec24p

The synthetic lethal interaction between Iss1p and several of the Sec24p mutants described above led us to screen our collection of alanine scanning mutants for similar synthetic lethal phenotypes. One mutant, Sec24R342A, was able to complement loss of Sec24p but was inviable in a Δsec24Δiss1 double mutant (Figure 7A). Sec24R342A was purified in complex with Sec23p.
Figure 7. Identification of a Second Cargo Binding Site on Sec24p

(A) Cells containing a deletion in the chromosomal copy of SEC24 (top) or in both SEC24 and ISS1 (bottom) were transformed with plasmids as indicated and grown on 5-FOA to cure cells of the plasmid containing wild-type SEC24. The Sec24R342A mutant was able to complement loss of SEC24, but was inviable in a Δsec24Δiss1 double mutant.

(B) In vitro budding reactions using Sec24R342A demonstrate a Sec22p-specific packaging defect.

(C) Surface representation of the Sec23/Sec24/Sar1 complex showing the location of the Bet1p/Sys1p site (Arg230, Arg235; purple) and the Sec22p site (Arg342; orange). This figure was generated using PyMOL (http://www.pymol.org).

and tested in in vitro budding reactions (Figure 7B). Packaging of the SNARE Sec22p was dramatically reduced in the presence of Sec24R342A (approximately 10% of wild-type). This packaging defect was specific for Sec22p; every other protein that we tested was packaged normally, including the other SNAREs (Sed5p, Bos1p, and Bet1p), as well as the p24 proteins (Erp1p and Emp24p), and the soluble cargo protein pro-α-factor (Figure 7B and data not shown). The location of Arg342 on the surface of Sec24p reveals that this second binding site is located on the same face of the protein as the Bet1/Sys1p binding site but further from the membrane and closer to Sec23p (Figure 7C). Together, the data presented here lead us to conclude that Sec24p has a number of independent cargo interaction domains that facilitate uptake of a diverse set of cargo molecules.

Discussion

Vesicular transport of proteins is a universal feature of eukaryotic cells, yet it represents a diverse process even within a single cell; different transport steps are mediated by distinct coats that must show unique cargo specificity. In furthering our understanding of the mechanisms that drive uptake of cargo molecules during vesicle formation, a combination of structural analysis and functional characterization of sites of interaction between sorting signals and cargo adaptor proteins has been particularly informative. Structural characterization of the interaction between tyrosine-based endocytic signals and the δ subunit of the clathrin AP-2 complex has not only revealed the physical basis for this interaction (Owen and Evans, 1998), but also allowed the identification of an additional novel binding determinant (Owen et al., 2001). Similar characterization of the acidic-cluster dileucine lysosomal sorting signal bound to the VHS domains of GGA proteins identified the residues critical for this interaction and demonstrated the requirement for those residues in efficient binding (Misra et al., 2002; Shiba et al., 2002). Here, we have described a detailed characterization of the mechanism of cargo binding by the COPII subunit, Sec24p, using a combina-
tion of biochemical and genetic approaches that provides insight into the molecular details of cargo uptake into ER-derived vesicles.

Interaction between cargo and coat proteins has been reconstituted using fusions of GST to cytoplasmic domains of cargo proteins. This approach has demonstrated that binding of some cargo molecules occurs directly to Sec24 (e.g., Sed5p; Peng et al., 1999); however, in some cases, binding has only been assessed for the Sec23/24p holo-complex. In our GST-Bet1p assay, Sec24p monomer is not recruited and binding of Sec23/24p requires the presence of Sar1p and a nonhydrolyzable GTP analog (Springer and Schekman, 1998). We note that a similar GST-Bet1p binding assay used by Mossessova et al. (2003) does not show this requirement for Sar1p, perhaps as a result of the 10-fold higher concentration of Sec23/24p used in their assay or the use of a truncated Sar1p that lacks the N-terminal helical domain. Full-length Sar1p binds directly to the cytosolic domain of Bet1p (Springer and Schekman, 1998) and in so doing may create a structure more favorable for interaction with Sec24p, or it may simply provide a binding site for Sec23p, the net effect of which would be to increase the avidity of Bet1p for Sec23/24p. Although the site of interaction between Bet1p and Sec24p has now been identified, the nature of the interaction between Bet1p and Sar1p and the physiological importance of such interaction remains to be further characterized.

We used the GST-Bet1p binding assay in combination with peptide competition to demonstrate that the binding site on Sec24p for Bet1p overlaps with that of Sys1p, a Golgi membrane protein that employs a diacidic sorting signal. Conversely, a peptide that interacts with Sec23/24p via a diaromatic motif is likely to bind to an independent site. That Bet1p and Sys1p occupy the same site on Sec24p was demonstrated by structural analysis of short peptides that correspond to the sorting signals of these two proteins (Mossessova et al., 2003).

Through a combination of a directed genetic screen and structural analysis, we were able to generate a series of mutations in Sec24p at the site occupied by the Bet1p and Sys1p sorting signals. In vitro budding and fusion experiments demonstrated that packaging of Bet1p was impaired with these Sec24p mutants, and consequently, vesicles were unable to undergo fusion with the Golgi apparatus. However, these in vitro data are not consistent with the in vivo phenotypes of most of these mutants, some of which displayed no obvious growth phenotype. How can we reconcile the viability of mutants that generate vesicles that fail to undergo downstream fusion? At least part of the answer to this question lies in the capacity for the Sec24p homologs Iss1p and Lst1p to compensate for defects in the Sec24p mutants. In the absence of both Iss1p and Lst1p, none of the mutants are capable of sustaining viability. The precise mechanism by which these two homologs compensate for most of the mutants, but not the more severely affected Sec24L616W, is unclear. It is likely that the different mutants have subtly different affinities for cargo molecules such that with a little help from the Sec24p homologs, cells are able to survive. More sensitive binding assays may allow better characterization of the affinities of these mutant proteins for a variety of cargo molecules.

In addition to Bet1p packaging defects, the Bet1p/Sys1p site mutants also showed impaired uptake of the SNAREs Bos1p and Sec22p, suggesting either a conservation of sorting motifs among these proteins or that the proteins are transported in a complex dependent on the Bet1p signal. Whereas Bos1p contains a sequence that is very similar to the Bet1p sorting motif (LVSLE), Sec22p contains no obvious homologous sequence. SNAREs are known to form complexes in cis, which must be disrupted by the action of NSF/Sec18p prior to trans-SNARE pairing (Wickner and Haas, 2000). We tested the possibility that a cis-SNARE complex conveys Sec22p by treating microsomal membranes with Sec18p prior to in vitro vesicle budding. However, no change in the packaging efficiency of Sec22p was observed (data not shown). Furthermore, the fact that a second, independent mutation in Sec24p specifically affects packaging of Sec22p suggests that this occurs via direct interaction with Sec24p, probably via a bipartite signal that makes contact with two sites on Sec24p. Like the Bet1p/Sys1p-site, the Sec22p binding site is well conserved between species, the exception being Lst1p, which is divergent at this site and does not package Sec22p (Miller et al., 2002). Clearly, the interaction of Sec22p with Sec24p warrants further characterization; structural analysis of the entire cytoplasmic domain of Sec22p in complex with Sec24p may be required to fully understand the nature of this complex interaction.

In an attempt to define the rules that govern cargo recognition, we surveyed a variety of cargo molecules, comparing vesicles made with wild-type Sec24p or Sec24L616W. One of the most surprising observations was that packaging of several amino acid permeases (Gap1p, Hip1p, and Can1p) was not impaired by mutation of the Bet1p/Sys1p binding domain on Sec24p. Gap1p contains a diacidic motif in its C-terminal cytoplasmic domain that is essential for efficient uptake into COPIII vesicles (Malkus et al., 2002). Further characterization of the contributions of the sorting sequences of Gap1p and Sys1p revealed that the Sys1p C terminus facilitated packaging of a truncated form of Gap1p into COPIII vesicles in a manner that depended on the Sys1p binding domain of Sec24p. Conversely, the Gap1p tail did not stimulate packaging of truncated Sys1p, suggesting that the Gap1p diacidic motif contributes to ER export in a distinct manner. One explanation for this discrepancy is that the Gap1p diacidic motif must be presented in a specific context to allow interaction with Sec24p. Such a context-specific requirement for sorting has recently been documented for the Erv41/46p complex, which contains two ER export signals that must each be presented in a specific orientation to facilitate efficient uptake into vesicles (Otte and Barlowe, 2002). In the case of Gap1p, packaging into COPIII vesicles is further complicated by the requirement for an accessory protein, Shr3p (Kuehn et al., 1996). Evidence to date suggests that the C-terminal domain of Gap1p does not mediate the interaction between Gap1p and Shr3p, and thus the Sys1p-Gap1p hybrid is unlikely to interact with Shr3p. If the Gap1p diacidic signal must be presented in the context of Shr3p to allow ER export, this trans-
planted signal would not function on Sys1p. Although the molecular details of Gap1p packaging remain to be further elucidated, it is clear that the Sys1p and Gap1p diacidic signals are distinct, suggesting that an additional domain of Sec24p is likely to interact with the Gap1p sorting signal. Unfortunately, we have been unable to detect an interaction between the Gap1p tail and Sec23/24p in vitro (P.N.M., unpublished data). Thus, a genetic screen selecting for Sec24p mutants that are impaired for Gap1p function may prove fruitful in identifying domains of Sec24p that are important for this cargo-coat interaction.

In addition to the SNAREs and diacidic sorting signals described above, we also examined cargo molecules with either unknown sorting signals or dihydrophobic/aromatic signals. Transport of the chitin synthase Chs3p was unaffected by mutation in the Sys1p binding pocket of Sec24p, suggesting an independent sorting motif. Chs3p is a large, mult spanning membrane protein that also requires an accessory protein, Chs7p, for efficient ER export (Trilla et al., 1999), but the sequence requirements for transport of either of these proteins are undefined. Likewise, a soluble luminal protein, α-factor, is presumed to enter COPII vesicles via interaction with its receptor, Erv29p (Belden and Barlowe, 2001b), but the regions of Erv29p that interact with Sec24p are not known. The final class of sorting signal that has been characterized in ER export is the dihydrophobic/aromatic signal. Packaging of these proteins with Sec24L616W ranged from unaffected (the Env41/46p complex, which contains both dihydrophobic and diaromatic signals) to marginally affected (Emp47p, exported in both Lst1p and Sec24p but which is slightly different in structure in Sec24p such that the affinity for Pma1p is reduced.

A tally of the cargo-interaction domains on Sec24p reveals three known sites: the Bet1p/Sys1p binding site and the Sec22p binding site described here, as well as the Sed5p site identified by Mossessova et al. (2003). Our analysis of the cargoes affected by mutations in the Bet1p/Sys1p site and Sec22p site suggest a number of cargo proteins bind to Sec23/24p independently of either region. Whether these proteins interact instead with the Sed5p site remains to be determined; however, it is possible that additional unidentified domains exist on Sec24p that further enrich the cargo diversity of COPII vesicles. Additional mutagenesis and functional analysis may reveal such sites, and more detailed characterization of the contents of vesicles generated with specific Sec24p and Lst1p mutants by mass spectrometry is likely to yield a more complete picture of the specificity of cargo recognition.

Experimental Procedures

Yeast Strains and Media

Strains used in this study are listed in Table 1. Cultures were grown at 30°C in either rich medium (YPD: 1% yeast extract, 2% peptone, 2% glucose) or synthetic complete medium (SC: 0.67% yeast nitrogen base, 2% carbon source, supplemented with amino acids as required). Complementation analysis was performed by streaking transformants onto SC-his supplemented with 5-fluoroorotic acid (5-FOA: 0.1% final concentration).

Plasmid Construction

Plasmids used in this study are listed in Table 1. Point mutations in Sec24 were introduced by QuickChange Mutagenesis (Stratagene) using primers containing the desired changes and plasmids pHM22 and pHM23 as template. Plasmids pHM22 and pHM23 contain a copy of the regions of Erv29p that interact with Sec23/24p independently of either region. Whether these proteins interact instead with the Sed5p site remains to be determined; however, it is possible that additional unidentified domains exist on Sec24p that further enrich the cargo diversity of COPII vesicles. Additional mutagenesis and functional analysis may reveal such sites, and more detailed characterization of the contents of vesicles generated with specific Sec24p and Lst1p mutants by mass spectrometry is likely to yield a more complete picture of the specificity of cargo recognition.

In Vitro Budding and Fusion

Microsomal membranes were prepared from wild-type cells (RSY620) or from a strain constitutively expressing a haemagglutinin-tagged version of Gap1p (Malkus et al., 2002) as described (Wuestehube and Schekman, 1992). Vesicle budding and fusion assays were performed...
formed as described (Miller et al., 2002). To assess transport of newly synthesized proteins, we prepared radiolabeled semi-intact cells and vesicle budding reactions were performed as described (Kuehn et al., 1996).

Microscopy

For electron microscopy, vesicles were generated from urea-washed microsomes as described above, using 1.5 mg of microsomal membranes in a 1 ml budding reaction containing 10 µg/ml Sar1p, 10 µg/ml Sec23/24p, or Sec23/24L616W and 20 µg/ml Sec13/31p. Vesicles in the medium-speed supernatant were fixed by addition of glutaraldehyde to a final concentration of 2%, collected by high-speed centrifugation at 100,000 × g, and the vesicle pellet processed further as described (Antonny et al., 2001). The diameters of 1000 vesicles were measured for each treatment and values given are the mean ± SD from ten different areas of each pellet.

Bet1p Binding Assays

GST-Bet1p was expressed in E. coli, purified, and used in binding assays essentially as described previously (Springer and Schekman, 1998). Briefly, GST-Bet1p was immobilized on glutathione-agarose beads and incubated with Sar1p (30 µg/ml) and Sec23/24p (30 µg/ml) in the presence of either GDP or GMP-PNP (0.1 mM) in binding buffer (20 mM HEPES [pH 6.8], 5 mM MgCl2, 1 mM EDTA, 300 mM KAc, 2% glycerol) in the presence of lipid emulsion (0.05% w/v PC/PE). Proteins were incubated for 1 h at 4°C to allow complex formation before immobilized complexes were washed extensively in binding buffer and eluted with SDS sample buffer for analysis by SDS-PAGE and SYPRO red staining. SYPRO-stained gels were scanned on a Typhoon 9400 Imager (Molecular Dynamics) and binding of Sec24p quantified using ImageQuant software (Molecular Dynamics). Peptide inhibition was performed by preincubating Sec24p with increasing concentrations of peptide in binding buffer at room temperature for 15 min before addition of Sar1p, nucleotide, lipid emulsion, and GST-Bet1p beads.

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