Distinct Roles for the Cytoplasmic Tail Sequences of Emp24p and Erv25p in Transport between the Endoplasmic Reticulum and Golgi Complex\

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Heteromeric complexes of p24 proteins cycle between early compartments of the secretory pathway and are required for efficient protein sorting. Here we investigated the role of cytoplasmically exposed tail sequences on two p24 proteins, Emp24p and Erv25p, in directing their movement and subcellular location in yeast. Studies on a series of deletion and chimeric Emp24p-Erv25p proteins indicated that the tail sequences impart distinct functional properties that were partially redundant but not entirely interchangeable. Export of an Emp24p-Erv25p complex from the endoplasmic reticulum (ER) did not depend on two other associated p24 proteins, Erp1 and Erp2p. To examine interactions between the Emp24p and Erv25p tail sequences with the COPI and COPII coat proteins, binding experiments with immobilized tail peptides and coat proteins were performed. The Emp24p and Erv25p tail sequences bound the Sec13p/Sec31p subunit of the COPII coat (Kd ~100 μM), and binding depended on a pair of aromatic residues found in both tail sequences. COPI subunits also bound to these Emp24p and Erv25p peptides; however, the Erv25p tail sequence, which contains a dilysine motif, bound COPII more efficiently. These results suggest that both the Emp24p and Erv25p cytoplasmic sequences contain a di-aromatic motif that binds subunits of the COPII coat and promotes export from the ER. The Erv25p tail sequence binds COPI and is responsible for returning this complex to the ER.

The secretory pathway in eukaryotic cells consists of a series of membrane-bound compartments that modify, sort and transport secretory cargo. Transport through this pathway depends on coat protein complexes that form vesicles and select specific cargo molecules for incorporation into vesicles. Current models suggest that transport between organelles is bi-directional, such that organelar constituents are recycled as secretory cargo advances. With regard to transport through the early secretory pathway, coat protein complex II (COPII) catalyzes anterograde transport between the ER and Golgi whereas coat protein complex I (COPI) acts in retrograde traffic between these compartments (1). In addition to coat-dependent export of secretory cargo from the ER, retrieval (2) and retention (3) mechanisms operate to maintain overall compartmental organization.

A related group of integral membrane proteins, referred to as the p24 family, are thought to act in concert with COPI and COPII to sort proteins during transport through the early secretory pathway. Initially identified on ER membranes (4) and subsequently detected as abundant proteins on COPI- and COPII-coated vesicles (5, 6), the function of p24 proteins in sorting remains unclear. In yeast strains lacking certain p24 members, some secretory proteins accumulate in the ER (e.g. invertase and the GPI-anchored protein Gas1p), while ER resident proteins that contain an HDEL retrieval motif are secreted and the unfolded protein response pathway is activated (6–9). Based on these and other findings, the p24 proteins have been proposed to act as structural components of vesicles (10), as cargo receptors (11), as negative regulators of vesicle budding (7) or to establish specialized subdomains on organelar membranes (12, 13). There are eight p24 proteins in yeast encoded by EMP24, ER25, and ERP1-ERP6 (14). Deletion of EMP24 and/or ER25 produce the strongest phenotypes with regard to the transport and sorting defects; however, deletion of the ERP1 and ERP2 genes also display similar but weaker phenotypes. Indeed, evidence suggests that Emp24p, Erv25p, Erp1p, and Erp2p function in a heteromeric complex (8, 14). There are no apparent phenotypes associated with deletion of the ERP3-ERP6 genes. Deletion of all eight p24-encoding genes in yeast produces viable cells with phenotypes that are indistinguishable from the single EMP24 or ER25 deletions (15).

The p24 proteins are composed of a luminal orientation amino-terminal domain and a single transmembrane segment that is followed by a cytoplasmically exposed ~12-amino acid carboxyl-terminal sequence. Many of the carboxyl-terminal tail sequences found in p24 proteins possess dilysine motifs that are predicted to interact with subunits of the COPI coat and localize these proteins to the early secretory pathway. Indeed, binding assays using immobilized tail sequences from p24 family members indicate a role for dilysine motifs in COPII binding and that a double phenylalanine sequence present from p24 family members is followed by a cytoplasmically exposed "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: COPI, coat protein complex II; ER, endoplasmic reticulum; COPI, coat protein complex I; PCR, polymerase chain reaction; kb, kilobase(s); SOE, splicing by overlap extension; PAGE, polyacrylamide gel electrophoresis; DMF, N,N-dimethylformamide; CB, coupling buffer; GPI, glycosylphosphatidylinositol.

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assays. Further, the coat-binding properties of these isolated tail sequences were determined. Our results suggest that both the Emp24p and Erv25p tail sequences interact with the COPII coat and direct this complex into COPII vesicles, whereas the Erv25p tail sequence is required in COPII binding and retrograde transport from the Golgi complex to the ER.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Growth Conditions**—Yeast strains used in this study are listed in Table I and were grown in rich medium (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose) or selective medium (0.67% nitrogen base without amino acids, 2% dextrose, and required supplements). Other standard media and genetic methods used have been previously described (19). The Escherichia coli strain DH5α was used for manipulation of recombinant DNA and was grown in LB medium (1% NaCl, 1% Bacto-trypcone, and 0.5% Bacto-yeast extract) containing 100 μg/ml ampicillin if required.

**Plasmid Construction**—All of the genes and gene fusion constructs used in this study were generated by polymerase chain reaction (PCR) amplification of DNA unless otherwise stated. PCR-amplified DNA was purified, cleaved, and ligated into appropriate vectors according to manufacturers specification. All constructs were sequenced to ensure that no errors were introduced during PCR amplification.

The ERV25 gene was subcloned from pBEV2 (8) into the yeast integrating vector pRS306 (20) using the restriction endonucleases PsI and EcoRI to produce pRV306. The EMP24 gene was obtained by PCR amplification of chromosomal DNA isolated from Saccharomyces cerevisiae strain FY834 (21) using oligonucleotides EM1 (5’-GGGAATTAGCGTACAAAGAGTTTCTG-3’) and EM2 (5’-CGGGATCCGTAAAAAG-TATGGAACCG-3’), which correspond to nucleotides –121 to –103 and nucleotides 719 to 701, respectively. The oligonucleotides EM1 and EM2 were engineered to contain the restriction endonuclease recognition sites EcoRI and BamHI, respectively, which allowed for convenient insertion into the yeast shuttle vector pRS114 (20) producing pREM314. Additional 5’-DNA sequence was added to pREM314 by subcloning a 1.2-kilobase (kb) XhoI/SalI fragment from pSEY-B22 (gift from H. Reisman) into the XhoI site, which is located in the multiple cloning region of pRS114, and the SalI site, which is located at nucleotide 394 in KMP24. An integrating version of this vector was constructed by transferring the 1.5-kb XhoI/BamHI fragment into pRS304 (20) to produce pRMP304.

The EME gene fusion was constructed using the gene Splicing by Overlap Extension (SOE) method (22). The 5’-portion of the gene fusion, termed Eme-A which spans amino acids 1–193 of Emp24p, was PCR-amplified using pRMP304 as a template with an oligonucleotide corresponding to the T3 promoter and TS2 (5’-GGTCTTAAGGTAG-TATATCGGAAAAAG-3’). The 3’ portion of the gene fusion, termed Eme-B which spans amino acids 202–211 of Erv25p, was PCR-amplified using pRVM306 as a template with oligonucleotides TS1 (5’TACTACT-TAAGACTACTTCAAAAGC-3’) and an oligonucleotide corresponding to the T7 promoter. The restriction endonuclease recognition site AluII was engineered into the homologous portion of TS1 and TS2 at the fusion junction. The SOE method was performed by mixing PCR-amplified fragments Eme-A and Eme-B with oligonucleotides TS1 and T7 followed by another round of amplification. The resulting 1.5-kb gene fusion was gel-purified and subcloned into pRS304 using the restriction endonucleases XhoI and BamHI generating pEME304.

The truncated EMP24 gene product, which lacks the 10-amino acid cytoplasmic tail, was made by first digesting the pEME304 vector with the restriction endonuclease AluII. Linearized DNA was gel-purified, and the 3’-recessed ends were filled-in with Klenow Fragment (New England Biolabs) and ligated. The fill-in reaction created a stop codon at amino acid 194, and the vector was termed pEMS304.

The EVE gene fusion was constructed using the SOE method. The 5’-portion of the gene fusion, termed Eve-A, which spans amino acids 1–201 of Emp24p was PCR-amplified using pRVM306 as a template with oligonucleotides EV1 (5’-GGGAATTAGCGTACAAAGAGTTTCTG-3’) and TS4 (5’TCTCCGGAGATGTCTGCCAACAC-3’). The 3’ portion of the gene fusion, termed Eve-B, which spans amino acids 194–203 of Emp24p, was PCR-amplified using pRMP304 as a template with oligonucleotides EM2 and TS3 (5’-AATCTTCGCCGATGTCTGGGATGGTCAAC-3’). The restriction endonuclease recognition site for BspEI was engineered into the homologous portion of TS3 and TS4 at the fusion junction. The SOE reaction was performed by mixing PCR-amplified fragments Eve-A and Eve-B with oligonucleotides EV1 and EM2. The resulting 1.0-kb gene fusion was subcloned into pRS306 using the restriction endonucleases EcoRI and BamHI producing pEVE306.

The ESV gene fusion also utilized the SOE method. The 5’-portion of the gene fusion termed EsvA was PCR-amplified using oligonucleotides EV1 and ESV2 (5’TATTACTAAGAGATGTCTGCAAC-3’) from pBEV2. The 3’-portion of the gene fusion, termed EsvB, was also PCR-amplified from pBEV2 using oligonucleotide ESV3 (5’TATTAG-TAATACCTTTCAAAACG-3’) and the 7T oligonucleotide. The SOE reaction was performed by mixing equal concentrations of EsvA and EsvB with the oligonucleotides EV1 and T7. The resulting 1.0-kb PCR-amplified DNA fragment was subcloned into pRS306 using the restriction endonucleases EcoRI and BamHI producing pEVS306.

**Strain Construction**—CBY244 (Δ24-Emp24p), CBY255 (Δ24-EME), and CBY289 (Δ24-EMS) were made by transforming CBY244 (Δ24) with pRMP304, pEME304, or pEMS304 after linearization with SnaBI for targeting to the trp1Δ63 locus. CBY241 (Δ25-Erv25p), CBY243 (Δ25-EVE), and CBY242 (Δ25-EMS) were constructed by transforming CBY114 (Δ25) with SthI-linearized pRVM306, pEVE306, or pEVS306 and targeted to the ura3-52 locus. CBY294 (Δ24/Δ25-EME-EVE) was made by first transforming CBY112 with linearized pEME304 and then

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<th>Strain</th>
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<td>CBY374</td>
<td>CBY294 with sec13–1</td>
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**TABLE I**

**Strains used in this study**
transforming with linearized pEVE306. An isogenic set strains expressing the chimeric tail fusions and the sec12–1 temperature sensitive allele was generated by repeated backcrosses of RSY265 (23).

Subcellular Fractionation—Sucrose gradient fractionation of membranes was performed as described with minor modifications (24). Homogenized spheroplasts were centrifuged at 450 × g in a Beckman SS34 rotor for 10 min, and 1 ml of the centrifuged cell lysate was layered on an 11-ml sucrose step gradient of 18, 22, 26, 30, 34, 38, 42, 46, 50, 54, and 60 (weight/volume) sucrose in 10 mM Hepes, pH 7.0, and 1 mM MgCl2. The gradients were centrifuged at 45,000 rpm in a Beckman Ti45 rotor for 2.5 h, and then 13 fractions of 770 μl were collected. A 1:1 dilution of the fractions in 2× sample buffer were analyzed by SDS-PAGE, then transferred to nitrocellulose, and probed using antibodies against Sec61p (25), Emp47p (26), Emp24p (6), and Erv25p (8).

Assays—Reconstituted COPII budding reactions, measurement of Kar2p secretion, and intracellular accumulation of Gas1p were performed as previously described by Belden and Barlowe (9).

COPII Interactions—Synthetic peptides corresponding to the 11 carboxyl-terminal amino acids of Emp24p and Erv25p with an amino-terminal cysteine residue were generated (Biosynthesis Inc. Louisville, TX) and linked to thiopropyl-Sepharose 6B (Pharmacia) as described (17) with minor modifications. Peptides with alanines substituted in Emp24p: FF-AA (CLRRAAETVSLV), and SLV-AAA (CLRRFFEVTAA) and Erv25p, YF-AA (CLKNAAKTKHII) were also generated. For the peptides corresponding to the Emp24p tails, the thiopropyl-Sepharose was swollen in 50% coupling buffer (CB) (0.1 M Tris-HCl, pH 7.5, 0.5 M NaCl) with 50% DMF (CB-DMF) for 15 min at room temperature. The beads were then washed two times in CB-DMF and brought up to a final volume of 80% beads in CB-DMF. The bead solution (500 μl) was transferred to a Microfuge tube with 2 mg of peptide and incubated overnight at room temperature with constant mixing. Coupled peptides were washed two times with CB-DMF and once with blocking buffer (0.1 M Tris-HCL, pH 7.5, 0.5 M NaCl, 5.0 mM 2-mercaptoethanol, 50% DMF) and then incubated for 30 min at room temperature in blocking buffer. The beads were washed four times in CB-DMF and brought up to final volume of 80% beads in CB-DMF. The synthetic peptides corresponding to the Erv25p tail were handled in a similar fashion. All CB-DMF was excluded. Peptide coupling efficiency was monitored by measuring the absorbance at 343 nm according to the manufacturer’s specifications. The amount of synthetic peptide bound to beads was quantified by a Lowery protein assay (27) using the synthetic peptides as standards.

In vitro binding reactions were performed in a 0.5-ml Microfuge tube for 1 h at 4 °C with mixing. Equal amounts of individual COPII subunits, a COPII mixture, or a crude cytosol were incubated with equal amounts of peptide coupled to beads in 100 μl of reaction buffer (150 mM KOAC, 10 mM Hepes, pH 7.0, and 0.1% Triton X-100). After incubation, the beads were transferred to a new 1.5-ml Microfuge tube and washed six times with reaction buffer. After the final wash, the remaining buffer was removed with a Hamilton syringe, and the beads were resuspended in 15 μl of 2× sample buffer. Samples (7 μl) were resolved by SDS-PAGE, and bound proteins were detected by immunoblot. After quantifying bound protein by densitometry, equilibrium dissociation constants (Kd) were calculated from double reciprocal plots of the data.

RESULTS

Rational—Multiple cytoplasmic targeting sequences are present on p24 complexes but it is not known how distinct sequences function in transport. We sought to define the functional roles of two specific tail sequences from Emp24p and Erv25p in directing movement of this p24 complex between the ER and Golgi compartments. A set of Emp24p and Erv25p deletion and tail swap chimeras were constructed and expressed at endogenous levels. These constructs were analyzed for complementation in vivo by monitoring Gas1p accumulation, Kar2p secretion, and suppression of sec12–1 temperature sensitivity. To assay more precisely the consequences of these alterations, we determined the subcellular distribution of these proteins and used a cell-free budding assay to measure their packaging efficiency into COPII vesicles. Finally, in vitro binding properties of Emp24p and Erv25p tail peptides with COPI and COPII subunits were determined.

Complementation Analyses of Emp24p/Erv25p Tail Deletions and Chimeras—An isogenic set of yeast strains expressing tail deletions and chimeras of the Emp24p-Erv25p complex were constructed as illustrated in Fig. 1. For example, the Δ24-EME strain (Emp24p with the Erv25p tail) expresses a chimeric EMP24p containing the COOH-tail of Erv25p in an emp24Δ strain. The Δ24-EMS strain (Emp24p with Stop codon at amino acid 194) expresses a truncated form of Emp24p lacking the final 10 amino acids at the carboxyl-terminal end in an emp24Δ strain. Both Δ24-EME and Δ24-EMS express an endogenous copy of ERV25. Similar approaches were used to generate the Δ25-EVE (Erv25p with Emp24p tail) and Δ25-EVS (Erv25p with stop codon) strains. Finally, the double swap strain (Δ/Δ-EME/EVE) carries emp24Δ and erv25Δ alleles and expresses chimeric proteins from constructs integrated at TRP1 and URA3.

Immunoblot analyses of membrane preparations from these strains were performed to assess the degree of complementation and the expression levels of the Emp24p and Erv25p proteins (Fig. 2). Yeast strains lacking a functional Emp24p-Erv25p complex accumulate the GPl-anchored secretory protein Gas1p in the ER as a 105-kDa species. In wild-type strains, mature Gas1p migrates as a fully glycosylated 130-kDa protein (6). As seen in the top panel of Fig. 2, no ER form of Gas1p was detected in the wild-type and Δ24-EME strains, whereas the other strains accumulated varying amounts of Gas1p in the ER. Denaturing scanning of these immunobots indicated that ~50% of the Gas1p contained in microsomes prepared from emp24Δ and erv25Δ strains migrated as the ER form. Similar levels were observed in the Δ25-EVE, Δ25-EVS, and Δ/Δ-EME/EVE strains. The Δ24-EMS strain accumulated an intermediate level of the ER form (~35%) suggesting partial complementation. These results are summarized in Table II.
Roles for the Cytoplasmic Tail Sequences of Emp24p and Erv25p

We also monitored the expression levels of the Emp24p and Erv25p proteins. As observed previously (8, 14) their expression was interdependent such that emp24Δ and erv25Δ reduced the levels of Erv25p and Emp24p, respectively. Near wild-type levels were observed in the Δ24-EME strain, and the other truncation or chimeric proteins were expressed at variably lower levels. Sec61p, an ER membrane protein that functions in polypeptide translocation, served as a loading control in these experiments to ensure equal distribution of samples.

Extracellular secretion of ER resident proteins (eg, Kar2p) is another phenotype associated with emp24Δ and erv25Δ strains (7). Therefore, we quantified the amount of Kar2p secreted from these strains after a 3-h growth period, and a representative experiment is shown in Fig. 3. Setting maximal Kar2p secretion levels at those observed for emp24Δ and erv25Δ, we found that the Δ25-EVS strain secreted near maximal levels, whereas Δ24-EMS, Δ25-EVE and Δ-Δ-EME,EVE secreted intermediate levels. The Δ24-EME strain secreted a low level of Kar2p that was near that of a wild-type strain.

We next determined if our set of EMP24 and ERV25 mutations could suppress the thermosensitivity of sec13–1 strains. Previous studies showed that deletion of EMP24 bypassed the requirement for SEC13 (7). Therefore, we crossed the sec13–1 mutation into our strains and scored for growth at 37 °C. As expected, both the emp24Δ and erv25Δ mutations suppressed sec13–1 (Table II). Interestingly, all of the mutations except Δ24-EME suppressed sec13–1 indicating that a partial loss of function mutation in EMP24 or ERV25 was adequate for suppression. All of these assays used to evaluate Emp24p/Erv25p function appear to show a close correlation; however, the Kar2p secretion assay seemed most sensitive followed by Gas1p accumulation then sec13–1 suppression. Based on these assays, we conclude that the EME fusion protein (Emp24p with the Erv25p tail) functioned at near wild-type levels and that the EMS and EVE proteins displayed partial function, whereas the EVS protein did not provide detectable activity. A complete swap of the Emp24p and Erv25p tail sequences (ΔΔ-EME/EVE) resulted in only modest complementation that was comparable with the ΔΔ-EVE strain (see Table II).

Subcellular Distribution and COPII Budding of Emp24p-Erv25p Deletions and Chimeras—Previous reports indicated that p24 proteins localize to membranes of the early secretory pathway (6, 8, 13, 17) and depend on their cytoplasmic tail sequences for proper localization (16, 18). To determine how the Emp24p and Erv25p tail sequences influence their steady state localization, we performed subcellular fractionation experiments on the set of tail deletions and chimera strains. Whole cell membranes were prepared from these strains and resolved on sucrose density gradients to separate ER and Golgi membranes (Fig. 4). Immunoblot analysis documented that a majority of a Golgi-localized marker protein (Emp47p) migrated in the upper portion of these gradients in fractions 3–7, whereas an ER resident protein (Sec61p) was found predominantly in fractions 9–11. We then measured the relative amounts of Emp24p and Erv25p found in these fractions to assess the percentage that localized to Golgi and ER membranes. These results are summarized in Table II. In a wild-type strain, ~43% of the Erv25p protein co-localized with Golgi membrane fractions and ~57% with ER containing fractions consistent with a previously reported distribution of Emp24p (6). In general, the tail deletion or chimeric strains displayed similar distribution patterns as the wild-type except the ΔΔ-EVE and ΔΔ-EME,EVE strains, which appeared to shift the Emp24p and Erv25p proteins toward Golgi membrane fractions. This shift to Golgi fractions seemed to correlate with loss of the Erv25p tail region. However, the complete erv25Δ deletion mutation caused a strong shift of residual Emp24p to the ER membrane fractions (Fig. 4E and Table II). We had previously observed that the erv25Δ mutation caused inefficient packaging of Emp24p into COPII vesicles (8), and this probably explains the shift of Emp24p to ER membrane fractions in the complete absence of Erv25p.

In all of these strains, a significant fraction of the Emp24p and Erv25p proteins co-fractionated with ER membranes. Therefore, we prepared ER membranes and measured the efficiency with which these proteins were packaged into COPII vesicles using a reconstituted budding assay (28). In this assay, washed microsomes were incubated with or without saturating amounts of purified COPII proteins (Sar1p, Sec23p-Sec24p complex, Sec13p-Sec31p complex) in the presence of GTP and an ATP regeneration system. Budded vesicles were then separated from microsomes by differential centrifugation, and their protein content compared with the total reaction (Fig. 5). As previously observed, the reconstituted budding reaction reproduces protein sorting during vesicle formation as Sec22p, an ER/Golgi SNARE protein, was efficiently packaged into COPII vesicles, whereas the ER resident protein Sec61p was not (28, 29). Importantly, COPII-dependent vesicle formation was efficient from all of the strains based on the packaging of Sec22p (Fig. 5 and Table II) and 35S-labeled gp-c-Factor (not shown). This result agreed with our previous finding that complete emp24Δ and erv25Δ deletion mutations did not influence overall COPII budding (8). Also consistent with our previous results, similar levels of Emp24p and Erv25p (8.8 and 8.4%, respectively) were packaged into COPII vesicles in a wild-type strain.

The percentage of Erv25p packaged into vesicles was near wild-type levels in all of the mutant strains except the ΔΔ-EME strain which was reduced by ~1/3. Even in an emp24Δ deletion strain, where Erv25p levels are severely reduced, this residual Erv25p was efficiently packaged into COPII vesicles (Table II). In contrast, Emp24p packaging efficiencies were significantly reduced in the ΔΔ-EME, ΔΔ-EVE and ΔΔ-EME,
Table II
Phenotypes of wild-type, p24 deletions, and chimeras

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Subcellular localization (percent ER: percent Golgi)
- Sec13p: 74%:26%
- Emp24p: 32%:68%
- Erv25p: 57%:43%

Emp24p: 77%:23%
Erv25p: 45%:55%

Incorporation into COPII vesicles (percent of total packaged)
- Sec22p: 12 13 11 11 13 11 11 12
- Emp24p: 77%:23% 45%:55%
- Erv25p: 62%:38%

Fig. 3. Strains expressing chimeric fusion proteins secrete varying amounts of Kar2p. A, extracellular Kar2p secreted to the cell culture supernatant was isolated by trichloroacetic acid precipitation then separated by SDS-PAGE followed by immunoblot analysis with antibodies specific for Kar2p. B, bar graph showing the relative amounts of Kar2p secreted to the cell culture supernatant as determined by densitometric scanning of the immunoblot.

EVE strains, where packaging efficiencies were ~1/2 of wild-type levels and similar to that observed for an erv25Δ deletion strain. These results correlate with the in vivo studies, in that efficient packaging of both Emp24p and Erv25p is necessary for functional complementation.

Erp1p and Erp2p Are Not Required for COPII Budding of Emp24p and Erv25p—Genetic and biochemical results indicate that Erp1p and Erp2p, additional p24 family members in yeast, interact with Emp24p and Erv25p (14). First, the erp1Δ and erp2Δ deletion strains display similar secretion defects as emp24Δ and erv25Δ strains. Second, the expression levels of Erp1p and Erp2p are interdependent and affect Emp24p and Erv25p expression. Third, co-immunoprecipitation experiments indicate a physical association between this subset of p24 proteins (14). The stoichiometry of this heteromeric p24 complex remains to be determined although a dimer of dimers has been proposed (30). Therefore we tested if Erp1p or Erp2p affect the packaging of Emp24p and Erv25p into COPII vesicles. ER membranes were prepared from an erv1Δ strain, a deletion that effectively removes both Erp1p and Erp2p (14). As seen in Fig. 6A, the erv1Δ strain accumulated the ER form of Gas1p and expressed diminished levels of Erv25p and Emp24p compared with a wild-type control. Next, reconstituted COPII budding reactions were performed from these membranes to measure the level of Emp24p and Erv25p packaging into vesicles (Fig. 6B). Even though the expression of Emp24p and Erv25p are lower in the erv1Δ membranes, the efficiency with which they were packaged into COPII vesicles was similar to the wild-type level. These data indicate that incorporation of the Emp24p and Erv25p proteins into COPII vesicles does not depend on Erp1p or Erp2p.

Coat Binding Properties of the Emp24p and Erv25p Tail Peptides—Our data indicate that both the Emp24p and Erv25p tail sequences are capable of directing these proteins into COPII-coated vesicles. To measure the binding properties between specific tail sequences and subunits of the COPII coat, we performed in vitro binding assay using peptides linked to Sepharose beads (17). In the first series of experiments, we determined if purified COPII subunits added at concentrations similar to those used to drive in vitro budding reactions could bind to the terminal 10 residues of the Emp24p (RRFFEVTSLV) and Erv25p (KNYFKTKHII). As shown in Fig. 7A, we detected binding of the Sec13p-Sec31p complex and the Sec23p-Sec24p complex to both tail sequences. In contrast, the Sar1p protein displayed specific binding to the Emp24p sequence and not to the Erv25p sequence. Under these conditions (~100-fold excess of peptide to protein), addition of COPII subunits individually or in a mixture did not alter their binding properties and furthermore, addition of guanine nucleotide (GTP or GTPγS) did not significantly alter binding (Fig. 7A). It seemed surprising that both the Sec23p-Sec24p complex and the Sec13p-Sec31p complex bound to these peptides so we performed additional experiments to address the affinity and specificity of these associations. The equilibrium dissociation constants between individual coat subunits and the Emp24p tail sequence were determined by varying the protein concentration and determining the amount of bound and free protein. The binding profiles for each protein (Fig. 7, B–D) was used to calculate the equilibrium dissociation constant for the Sec13p-Sec31p complex ($K_d = 120 \mu M$), the Sec23p-Sec24p complex ($K_d = 600 \mu M$) and for Sar1p ($K_d = 130 \mu M$). Therefore, under these condi-
The Sec13p-Sec31p complex displayed the highest binding affinity followed by Sar1p then the Sec23p-Sec24p complex. To identify specific residues in the Emp24p and Erv25p tail peptides necessary for binding to COP II subunits, we changed conserved amino acids to alanines, then monitored COP II binding (Fig. 8A). A pair of aromatic amino acid residues at positions 7 and 8 in both the Emp24p and Erv25p sequences were necessary for binding of the Sec13p-Sec31p complex. These residues (FF) in the Emp24p sequence were also critical for Sar1p binding. It has been reported that the terminal two

Fig. 4. Subcellular fractionation on sucrose gradients of strains expressing chimeric fusion proteins. Whole cell lysates were separated on 18–60% (weight/volume) sucrose density gradients and fractions were collected from the top. Relative amounts of Sec61p, Emp47p, Erv25p and Emp24p in each fraction were quantified by densitometry of immunoblots. Percent sucrose of each fraction was determined using a refractometer. A, FY834 (WT), B, CBY99 (Δ24), C, CBY245 (Δ24-EME), D, CBY289 (Δ24-EMS), E, CBY114 (Δ25), F, CBY243 (Δ25-EVE), G, CBY242 (Δ25-EVS), and H, CBY294 (Δ24/Δ25-EME, EVE).
Roles for the Cytoplasmic Tail Sequences of Emp24p and Erv25p

Previous reports indicated that the cytoplasmic tail sequences contained on p24 proteins are important for their localization and can mediate associations with the COPI and COPII coat complexes (13, 16–18). Here we focus on the Emp24p and Erv25p tail sequences in yeast to determine how they direct the movement and location of this p24 complex in the early secretory pathway. Our in vivo analyses and in vitro assays to monitor function indicate that these tail sequences impart distinct functional properties that are partially redundant but not entirely interchangeable. Based on our findings, we propose that both the Emp24p and Erv25p cytoplasmic sequences contain a di-aromatic motif that binds directly to subunits of the COPII coat and promotes export from the ER. The Erv25p tail sequence possesses an additional di-lysine motif that assists in binding subunits of the COPI coat and is required for returning this complex to the ER.

When the Emp24p and Erv25p tail sequences were deleted or exchanged we observed redundant functional information contained in the Emp24p tail. Specifically, replacement of the Emp24p tail with the corresponding Erv25p cytoplasmic sequence (Δ24-EME) resulted in essentially normal operation of this complex. The Δ25-EVE mutation did not suppress the sec13-1 temperaure-sensitive mutation and even bypasses a complete deletion of SEC13 (7). As an additional test of function, we found that the Δ24-EME mutation did not suppress sec13-1 as ex-

ever the level of binding was significantly higher when the Erv25p tail was used and probably correlated with the presence of a di-lysine motif (KTKHII) found in this sequence. Conversion of the aromatic residues at −7 and −8 to alanines in the Erv25p sequence decreased the amount of COPI that bound and suggested that maximal COPI binding depends on both the di-lysine motif and the pair aromatic residues.

DISCUSSION

We next tested the COPI binding properties of these peptides using a clarified cytosol as a source of COPI protein. After incubations, beads were washed, and the amount of COPI bound to peptides detected using an antiserum prepared against purified yeast COPI (32). As seen in Fig. 8, both the Emp24p and Erv25p tail peptides bound COPI compared with the control beads. How-
expected for a functional Emp24p-Erv25p complex, whereas the Δ25-EVE mutation suppressed thermosensitivity. Additionally, the Δ24-EMS and Δ3-EME,EVE mutants, which displayed varying levels of function as assessed by the assays that measure Gas1p accumulation and Kar2p secretion, did not suppress the sec13–1 mutation. These results indicate that even partial loss of function mutations in EMP24 or ERV25 are sufficient for SEC13 bypass.

To monitor the efficiency with which the chimeric and deletion complexes were exported from the ER, we performed reconstituted budding assays in vitro. In wild-type and Δ24-EME membranes, both the Emp24p and Erv25p proteins were packaged with equal efficiency into COPII vesicles. In the other mutants, varying levels of packaging were observed but none packaged both Emp24p and Erv25p at wild-type levels. Interpretation of these results is complicated because mislocalization and/or instability of the mutants influence our ability to accurately measure export efficiency. In some instances, expression levels and localization of the Emp24p-Erv25p complex were related to levels of packaging, although a strict correlation was not observed.

The Emp24p-Erv25p complex does not depend on Erp1p and Erp2p for export from the ER. Cell-free budding assays from a strain lacking Erp1p reveal that Emp24p and Erv25p continue to be efficiently recruited into COPII vesicles. Previous results indicated that Emp24p, Erv25p, Erp1p, and Erp2p form a heteromeric complex and that all four subunits are required for optimal expression levels (14). Our results are in accord with these findings as we observed that erp1Δ caused a reduction in the expression level of the Emp24p-Erv25p complex. However,
Roles for the Cytoplasmic Tail Sequences of Emp24p and Erv25p

the remaining Emp24p-Erv25p complex in an erpΔ strain retained function and was efficiently packaged into COPII vesicles. In contrast, Erp1p and Erp2p expression and function appear to depend on the Emp24p and Erv25p proteins (14). Our results suggest that a core heterodimer consisting of Emp24p-Erv25p forms and that some of this dimer associates with Erp1p and Erp2p to form a heterotetramer. The heteromeric associations are probably mediated by heptad repeat regions conserved among p24 family members (30). These results are also consistent with a study in mammalian cells indicating that heteromeric p24 proteins exist in dynamic equilibrium with multiple family members (33). We conclude that optimal expression of the Emp24p-Erv25p complex depends on Erp1p and Erp2p but that the targeting information contained in the cytoplasmic tail sequences of Emp24p and Erv25p is sufficient for transport between the ER and Golgi compartments.

We also examined the direct interactions between purified subunits of the COPII coat and the Emp24p and Erv25p tail peptides. Both the affinity and specificity of these interactions were assessed. Surprisingly, all subunits of the COPII coat displayed some affinity for the Emp24p tail peptide compared with control beads. Binding of individual COPII subunits to an excess of the Emp24p tail peptide appeared to be independent because equal binding was observed when proteins were added individually or as a mixture. In other words, binding of the Sec23p-Sec24p complex and Sec13p-Sec31p complex did not require initial binding of Sar1p-GTP as observed for other COPII vesicle cargo proteins (35). However, the affinities of these direct interactions were distinct. The Sec13p-Sec31p complex and Sar1p exhibited equilibrium dissociation constants in the 100-μM range, whereas this constant was 5-fold greater for Sec23p-Sec24p complex. Furthermore, conversion of the diphenylalanine residues at positions 7 and 8 abolished binding of the Sec13p-Sec13p complex and Sar1p but not Sec23p-Sec24p. These findings suggest that the Emp24p tail sequence interacts with Sar1p and Sec13p-Sec31p complex during export from the ER. The Erv25p tail peptide also bound the Sec13p-Sec31p complex and Sec23p-Sec24p complex but not Sar1p. As with the Emp24p tail peptide, Sec13p-Sec31p binding depended on a pair of aromatic residues at positions 7 and 8; however, in this instance the residues are YF instead of FF. Because Sar1p binding to the Emp24p tail depended on the FF residues, these results indicate that the FF motif is critical for Sar1p association and the YF residues could not substitute, at least in these in vitro binding experiments. Previous reports in mammalian cells described the binding of specific cytoplasmic tail peptides with the Sec23p protein from whole cell lysates (18, 34). Our findings are not entirely consistent with these studies; however, significantly different approaches were employed.

An affinity for COPII subunits probably acts to recruit the Emp24p-Erv25p complex to vesicle formation sites on the ER membrane. However, in the case of Emp24p tail sequence, it seems unlikely that Sar1p and Sec13p-Sec31p could bind simultaneously to this short motif. Therefore binding may be sequential such that Sar1p binds initially to attract the Sec23p-Sec24p complex and then the Sec13p-Sec31p complex. For example, after GTP hydrolysis by Sar1p, a coat structure may persist for some period of time through an association between p24 tail sequences and the Sec13p-Sec31p complex. Alternatively, forming vesicles could contain an excess of p24 proteins and an association between Sec13p-Sec31p complex, and the Emp24p or Erv25p tail sequences may serve a regulatory role in controlling the GTPase rate of Sar1p. A recent report indicates that binding of Sec13p-Sec31p stimulates the Sec29p-Sec42p GAP activity toward Sar1p (36), therefore the recruitment of Sec13p-Sec31p by p24 proteins could influence the rate of GTP hydrolysis by Sar1p. Such a regulatory role may also explain how deletions of Emp24p or Erv25p bypass loss of function mutations in the Sec13p subunit of COPII (7).

Finally, the Erv25p tail sequence, which contains lysine residues at positions 4 and 6, bound subunits of the COPI coat at an increased level compared with the Emp24p tail. Even after changing the YF to AA, the Erv25p tail was still capable of binding COPII subunits although with slightly reduced efficiency. Together, these findings support a model whereby the Emp24p tail operates in COPII binding and anterograde movement, whereas the Erv25p tail sequence associates with both COPI and COPII coats and operates in anterograde and retrograde movement of this p24 complex between the ER and Golgi.

REFERENCES