Efficient export of secretory alkaline phosphatase (ALP) from the endoplasmic reticulum depends on the conserved transmembrane sorting adaptor Erv26p/Svp26p. In the present study we investigated the mechanism by which Erv26p couples pro-ALP to the coat protein complex II (COPII) export machinery. Site-specific mutations were introduced into Erv26p, and mutant proteins were assessed in cell-free assays that monitor interactions with pro-ALP cargo and packaging into COPII vesicles. Mutations in the second and third loop domains of Erv26p inhibited interaction with pro-ALP, whereas mutations in the C-terminal tail sequence influenced incorporation into COPII vesicles and subcellular distribution. Interestingly mutations in the second loop domain also influenced Erv26p homodimer associations. Finally we demonstrated that Ktr3p, a cis-Golgi-localized mannosyltransferase, also relies on Erv26p for efficient COPII-dependent export from the endoplasmic reticulum. These findings demonstrate that Erv26p acts as a protein sorting adaptor for a variety of Type II transmembrane cargo proteins and requires domain-specific interactions with both cargo and coat subunits to promote efficient secretory protein transport.

Anterograde transport in the eukaryotic secretory pathway is initiated by the formation of COPII-coated vesicles that emerge from transitional ER sites. The COPII coat, which consists of the small GTPase Sar1p, Sec23/24 complex, and Sec13/31 complex, selects vesicle cargo through recognition of export signals and forms ER-derived vesicles through assembly of an outer layer cage structure (1, 2). Cytoplasmically exposed ER export signals have been identified in secretory cargo including the C-terminal dihydrophobic and diacidic motifs (3, 4). Structural studies indicate that the Sec24p subunit of the COPII coat contains distinct binding sites for some of the molecularly defined export signals (5, 6). Thus a cycle of cargo–coat interactions regulated by the Sar1p GTPase directs anterograde movement of secretory proteins into ER-derived transport vesicles (7).

Although many secretory proteins contain known export signals that interact directly with COPII subunits, the diverse array of secretory cargo that depends on this export route requires additional machinery for efficient collection of all cargo into COPII vesicles (1). For instance soluble secretory proteins as well as transmembrane cargo require protein sorting adaptors for efficient ER export. These membrane-spanning adaptors, or sorting receptors, interact directly with secretory cargo and with coat subunits to efficiently couple cargo to the COPII budding machinery. For example, ERGIC-53 acts as a protein sorting adaptor for several glycoproteins and has a large N-terminal luminal domain that interacts with secretory proteins including blood coagulation factors, cathepsins, and α1-antitrypsin (8–10). The cytoplasmic C-terminal tail of ERGIC-53 contains a diphenylalanine export signal that is necessary for COPII export as well as a dilsyline motif required for COPII-dependent retrieval to the ER (11). Additional ER vesicle proteins identified in yeast have been shown to interact with the COPII coat as well as specific secretory proteins (12). For example Erv29p acts as a protein sorting adaptor for the soluble secretory proteins glyco-pro-α-factor and carboxypeptidase Y (13). Erv29p also contains COPII and COPII sorting signals that shuttle the protein between ER and Golgi compartments. More recently Erv26p was identified as a cargo receptor that escorts the pro-form of secretory alkaline phosphatase (ALP) into COPII-coated vesicles (14).

Although COPII sorting receptors have been identified, the molecular mechanisms by which these receptors link cargo to coat remain poorly understood. Moreover it is not clear how cargo binding is regulated to promote interaction in the ER and then trigger dissociation in the Golgi complex. We have shown previously that Erv26p binds to pro-ALP and is required for efficient export of this secretory protein from the ER (14). Therefore specific luminal regions of Erv26p are proposed to interact with pro-ALP, whereas cytosolically exposed sorting signals are presumably recognized and bound by coat subunits. To gain insight on the molecular contacts required for Erv26p sorting function, we undertook a systematic mutational analysis of this multispanning membrane protein. After generating a series of Erv26p mutants, we observed that mutation of specific residues in the third loop domain affect pro-ALP interaction and that residues in the C-terminal cytosolic tail are required for COPII and COPI transport. Finally mutation of residues in the second loop domain influenced Erv26p homodimer formation and sorting activity.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media**—Strains used in this study are listed in Table 1. Cells were grown in rich medium (1% Bacto yeast extract, 2% Bacto peptone, 2% dextrose) or minimal medium.
Molecular Dissection of Erv26p

TABLE 1
Yeast strains used in this study

| Strain       | Genotype                          | Source or Ref.    |
|--------------|-----------------------------------|------------------|
| CBY740       | MATa his3 leu2 lys2 ura3           | Research Genetics BY4742 |
| CBY1480      | MATa his3 leu2 lys2 ura3 erv26ΔKAN* | Research Genetics BY4742 |
| CBY1912      | CBY1480 with pRS316-ERV26         |                  |
| CBY1917      | MATA his3 leu2 lys2 ura3 HIS3MX6-Gal1-3XHA-ERV26 | This study |
| CBY2150      | MATa his3 leu2 lys2 ura3 Erv26-3HA-HIS3MX6 | This study |
| CBY2176      | MATa his3 leu2 lys2 ura3 PHO8-3XHA-HIS3MX6 | This study |
| CBY2305      | CBY2150 with pRS316-ERV26               |                  |
| CBY2462      | MATa his3 leu2 lys2 ura3 PHO8-3XHA-HIS3MX6 erv26ΔKAN* with pRS316-ERV26 | This study |
| CBY2891      | CBY1480 with pRS316-ERV26-LLEL2-5AAAAA | This study |
| CBY2617      | CBY1480 with pRS316-ERV26-LLEL2-3AA  | This study |
| CBY2616      | CBY1480 with pRS316-ERV26-L3E      |                  |
| CBY2366      | CBY1480 with pRS316-ERV26-E4A      |                  |
| CBY2618      | CBY1480 with pRS316-ERV26-LLL5-6AA  |                  |
| CBY2367      | CBY1480 with pRS316-ERV26-E22A     |                  |
| CBY2315      | CBY1480 with pRS316-ERV26-EE35-36AA |                  |
| CBY2368      | CBY1480 with pRS316-ERV26-RR42-43AA |                  |
| CBY2596      | CBY1480 with pRS316-ERV26-KL67-68AT |                  |
| CBY2595      | CBY1480 with pRS316-ERV26-K67N     |                  |
| CBY2628      | CBY1480 with pRS316-ERV26-S72A     |                  |
| CBY2597      | CBY1480 with pRS316-ERV26-I73N     |                  |
| CBY2303      | CBY1480 with pRS316-ERV26-C75M     |                  |
| CBY2342      | CBY1480 with pRS316-ERV26-FS71-73AAA | This study |
| CBY2308      | CBY1480 with pRS316-ERV26-YIVYYVY76-80AAAAA | This study |
| CBY2629      | CBY1480 with pRS316-ERV26-Y76A     |                  |
| CBY2460      | CBY1480 with pRS316-ERV26-YY79-80AA |                  |
| CBY2631      | CBY1480 with pRS316-ERV26-L83N     |                  |
| CBY2598      | CBY1480 with pRS316-ERV26-Q81A     |                  |
| CBY2460      | CBY1480 with pRS316-ERV26-N82G     |                  |
| CBY2599      | CBY1480 with pRS316-ERV26-K84A     |                  |
| CBY2892      | CBY1480 with pRS316-ERV26-NH106-107AA | This study |
| CBY2369      | CBY1480 with pRS316-ERV26-H107A    |                  |
| CBY2317      | CBY1480 with pRS316-ERV26-YF114-115AA | This study |
| CBY2365      | CBY1480 with pRS316-ERV26-K112A    |                  |
| CBY2600      | CBY1480 with pRS316-ERV26-F14A     |                  |
| CBY2601      | CBY1480 with pRS316-ERV26-D116A    |                  |
| CBY2602      | CBY1480 with pRS316-ERV26-E118A    |                  |
| CBY2316      | CBY1480 with pRS316-ERV26-P120A    |                  |
| CBY2603      | CBY1480 with pRS316-ERV26-Q122A    |                  |
| CBY2604      | CBY1480 with pRS316-ERV26-K124A    |                  |
| CBY1996      | CBY1480 with pRS316-ERV26-E218A    |                  |
| CBY1997      | CBY1480 with pRS316-ERV26-E218A-E220A |                  |
| CBY1995      | CBY1480 with pRS316-ERV26-D222A    |                  |
| CBY1994      | CBY1480 with pRS316-ERV26-D222A-D244 |                  |
| CBY2893      | CBY1480 with pRS316-ERV26-RKYIYLSL205-211AA       |                  |
| CBY2894      | CBY1480 with pRS316-ERV26-RRVINSV198-204AAAAAAA     |                  |
| CBY2895      | CBY1480 with pRS316-ERV26-VV199-200TT         |                  |
| CBY2896      | CBY1480 with pRS316-ERV26-V228D        |                  |
| CBY2897      | CBY1480 with pRS316-ERV26-ΔRALV (residues 225–228) | This study |
| CBY2047      | MATa his3 leu2 lys2 ura3 Erv26ΔC1(173–228)-3HA-HIS3MX6 | This study |
| CBY2847      | MATa his3 leu2 lys2 ura3 Erv26ΔC2(162–228)-3HA-HIS3MX6 | This study |
| CBY2548      | MATa his3 leu2 lys2 ura3 KTR3-3XHA-HIS3MX6 | This study |
| CBY2767      | MATa his3 leu2 lys2 ura3 KTR3-3XHA-HIS3MX6 erv26ΔKAN* with pRS316-ERV26-P120A | This study |
| CBY2768      | MATa his3 leu2 lys2 ura3 KTR3-3XHA-HIS3MX6 erv26ΔKAN* with pRS316-ERV26-Q122A | This study |
| CBY2322      | MATa his3 leu2 lys2 ura3 PHO8-3XHA-HIS3MX6 erv26ΔKAN* with pRS316-ERV26-P120A | This study |
| CBY2622      | MATa his3 leu2 lys2 ura3 PHO8-3XHA-HIS3MX6 erv26ΔKAN* with pRS316-ERV26-Q122A | This study |
| CBY2319      | CBY2150 with pRS316-ERV26-P120A     |                  |
| CBY2755      | MATa his3 leu2 lys2 ura3 PHO8-3XHA-HIS3MX6 erv26ΔKAN* with pRS316-ERV26-C75M | This study |
| CBY2758      | MATa his3 leu2 lys2 ura3 PHO8-3XHA-HIS3MX6 erv26ΔKAN* with pRS316-ERV26-C75M | This study |
| CBY2762      | CBY2150 with pRS316-ERV26-C73N     |                  |
| CBY2768      | CBY2150 with pRS316-ERV26-C75M     |                  |

(0.67% yeast nitrogen base without amino acids, 2% dextrose) with appropriate supplements at 30 °C unless otherwise noted. Standard yeast (15) and bacteria (16) molecular genetic methods were used.

Plasmid Construction—Construction of pRS316-ERV26 was described previously (14). Mutagenesis of pRS316-ERV26 was performed using the QuickChange site-directed mutagenesis method (Stratagene), and mutants were verified by sequencing. Oligonucleotide primers used in this study are available upon request.

Strain Construction—The erv26Δ::KAN and ALP-HA strains have been described previously (14). Ktr3-HA was constructed by targeting the KTR3 gene with the PCR product generated from pFA6a-3HA-His3MX6 (17) using primers Ktr3-HA.F2 and Ktr3-HA.R1. Erv26-HA was constructed using the same method targeting the ERV26 gene with the PCR product of pFA6a-3HA-His3MX6 using primers Erv26-HA.F2 and Erv26-HA.R1. HA-Erv26 was constructed by targeting the ERV26 gene with the PCR product of pFA6a-His3MX6-PGAL1-3HA using primers YHR181W-F4 and YHR181W-R3.

Antibodies and Immunoblotting—Antibodies directed against Erv25p (18), Sec61p (19), Kar2p, Och1p, Erv41p, Erv46p (12), Sec22p (20), ALP (21), and Erv26p (14) have been described previously. Immunoblots were developed using SuperSignal.
West Pico detection reagents (Thermo Scientific) and developed with a UVP bioimaging system (Upland, CA).

**Membrane Preparations**—Yeast semi-intact cell membranes (22) and microsomal membranes (23) were isolated as described previously. Subcellular fractionation of organelles was performed as described previously (14).

**Protease Protection Assay**—Microsomes (0.75 mg of membrane protein/ml final) in Buffer 88 (20 mM HEPES, pH 7.0, 250 mM sorbitol, 150 mM KOAc, 5 mM MgOAc) were mixed with or without proteinase K (final concentration, 0.2 mg/ml). Where noted, 0.2% Triton X-100 was included. Reactions were incubated for 40 min on ice and stopped by addition of 2 mM phenylmethylsulfonyl fluoride. One volume of 5 × SDS-PAGE sample buffer was added, and proteins were resolved on 12.5% polyacrylamide gels for immunoblot analysis.

**Chemical Cross-linking**—Microsomes (0.85 mg of membrane protein/ml final) were mixed with Buffer 88 and increasing concentrations of dithiobissuccinimidyl propionate (DSP) in dimethyl sulfoxide (0.1–0.4 mM DSP) or DMSO alone and incubated for 20 min at 20 °C. Cross-linking was quenched by incubation with 2.5 mM Tris, 20 mM glycine on ice for 10 min. Reactions were resolved on polyacrylamide gels and analyzed by immunoblot.

**In Vivo Labeling**—Pulse-chase experiments were performed as described previously (18). Briefly cells were grown in reduced sulfate media to an A600 of 0.4, harvested, washed and resuspended at an A600 of 3.0 in minimal media without sulfate. After preincubation at 30 °C for 5 min, cultures were pulsed with 35S-PRO-MIX (Amersham Biosciences) at 25 Ci/μg. The chase phase was initiated after 7 min by the addition of excess unlabeled methionine and cysteine. ALP and carboxypeptidase Y were immunoprecipitated from common extracts and resolved on 8% polyacrylamide gels, and labeled proteins visualized by phosphorimaging (GE Healthcare). To estimate half-times, the percentage of labeled ALP that had been processed to the mature form was plotted over time.

**Immunoprecipitation of Erv26p Complexes**—Immunoprecipitation experiments were performed as described previously (14) with the following modifications. Wild-type microsomes or semi-intact cells (220 μg of total membrane protein in 225 μl) were solubilized in an equal volume of Buffer 88, pH 8.0 containing 2% digitonin (B88-8/2.0% digitonin) or buffer 88, pH 7.0 containing 2% Triton X-100 (B88-2.0% TX100) as indicated in the presence of 1 mM phenylmethylsulfonyl fluoride and 5 mM EDTA. Digitonin-containing samples were centrifuged at 14,000 rpm for 2 min at room temperature, and Triton X-100-containing samples were centrifuged at 60,000 rpm for 10 min at 4 °C to pellet unsolubilized material. The supernatant fluid (~400 μl) was recovered, and 5% of this solubilized material was saved as a total sample. The remainder was diluted with 2 volumes of B88-8/0.5% digitonin or B88/0.2% TX100. To immunoprecipitate ALP-HA, Ktr3-HA, or Erv26-HA, 0.25 μg of the monoclonal HA.11 antibody (Sigma-Aldrich) and 40 μl of 20% protein A-Sepharose beads were added. To immunoprecipitate Erv26p, 0.25 μg of the polyclonal Erv26p antibody and 40 μl of 20% protein A-Sepharose beads were added. After binding for 70 min at 4 °C, immunocomplexes bound to Sepharose beads were washed four times with B88-8/0.5% digitonin or B88/0.2% TX100. Bound proteins were eluted from the beads by adding 20 μl of SDS-PAGE sample buffer followed by incubation at 75 °C for 3 min. Total and immunoprecipitated samples were resolved on polyacrylamide gels and analyzed by immunoblot. Recoveries were calculated based on densitometric analysis using the Image J program. Representative results are shown after multiple replicate experiments.

**In Vitro Budding Assays**—Large scale in vitro budding was performed with microsomes in the absence or presence of purified COPII components (24). A 22-μl portion of total reactions and 220 μl of supernatant fractions containing vesicles were centrifuged at 60,000 rpm (TLA100.3 rotor, Beckman Instruments) to pellet membranes. Membrane pellets were resuspended in SDS-PAGE sample buffer, and equal volumes were loaded on 12.5% polyacrylamide gels for immunoblots. Where indicated, preimmune or anti-Erv26p antibodies were added to membranes and incubated on ice for 15 min prior to the addition of budding factors. Packaging efficiencies were calculated as a percentage of totals by densitometry.

**RESULTS**

**Erv26p Topology**—Erv26p is a non-essential transmembrane protein that is predicted to contain four transmembrane segments (25, 26) with cytosolic N and C termini. To test this arrangement, location of the N and C termini were examined. We had previously performed in vitro budding experiments in the presence of a polyclonal Erv26p antiserum, which was raised against the C-terminal 69 amino acid residues of Erv26p, and observed that these antibodies specifically inhibited uptake of Erv26p into COPII vesicles (14). This result suggested that the C-terminal tail of Erv26p faces the cytosol. To investigate topology, two epitope-tagged versions of Erv26p were generated, placing 3XHA tags on either the N or C terminus at the ERV26 locus (17). The tagged proteins were stably expressed and supported pro-ALP processing to near wild-type levels (Fig. 4 and data not shown). Microsomal membranes were prepared from both HA-Erv26 and Erv26-HA strains and then used in protease protection assays to examine the accessibility of epitope-tagged Erv26p to proteinase K in the absence and presence of detergent.

As seen in Fig. 1A, proteinase K treatment of HA-Erv26p in the absence of detergent (lane 3) caused a marked reduction in HA-tagged protein but also produced a partially protected species that was shifted in size by ~8 kDa when immunoblots were developed with anti-HA. This proteolytic product was not detected with the C terminus-specific Erv26p polyclonal antisera and the size shift corresponds to the approximate size of the 69-amino acid C terminus. In the presence of detergent, HA-Erv26p was completely digested, and no partial digestion products were observed with either antibody (lane 4). These results indicate that the N-terminal HA tag was largely sensitive to protease treatment in intact microsomal membranes; however, a minor protease-protected fragment was also generated from HA-Erv26.

Proteinase K treatment of C-terminally tagged Erv26-HA membranes (Fig. 1A, lane 7) digested virtually all Erv26-HA protein as detected in both anti-HA and anti-Erv26p immunoblots. Residual Erv26-HA was completely digested in the pres-


Molecular Dissection of Erv26p

![Diagram](Image)

**FIGURE 1.** Erv26p N and C termini face the cytoplasm. A, microsomes were treated with 0.2 mg/ml proteinase K (Prot K) with or without 0.2% Triton X-100 (TX-100) for 40 min on ice. Proteinase K activity was inhibited by addition of 2 mM phenylmethylsulfonyl fluoride, and reactions were analyzed by immunoblot with the indicated antibodies. Samples contained no addition (lane 1), detergent alone (lane 2), proteinase K (lane 3), or detergent and proteinase K (lane 4). The arrowhead indicates the position of a partial protease-protected species detected after protease treatment of HA-Erv26p-containing membranes. B, proposed model of Erv26p indicating conserved residues, topology, and loop domain mutations. A ClustalW (1.83) alignment was performed using the Erv26p sequence (gi:6321975) and homologous sequences from human (gi:37182685), mouse (gi:13277843), Drosophila melanogaster (gi:18446873), and Schizosaccharomyces pombe (gi:3080515) in the GenBank™ Database. Invariant residues (orange) are identical in all five species, whereas conserved residues (yellow) are identical across three or four species. The topology model places the N and C termini in the cytoplasm with residues in Loop I and Loop III within luminal compartments. Numbers indicate amino acid position in the protein. Mutations in Loop III (arrowhead) influence Erv26p interactions with pro-ALP, whereas deletion of the C-terminal cytosolic tail influences COPII packaging and intracellular localization. Mutation of residues in Loop II (arrow) influences homodimer stability and cargo interactions.

ence of detergent (lane 8). These observations indicate that the C-terminal HA tag was also sensitive to proteinase K and therefore was cytosolically exposed, consistent with previous evidence for antibody accessibility to the C terminus of Erv26p (14). As controls for membrane integrity and proteinase K activity in these experiments, Erv46p, a transmembrane protein with relatively short cytosolic segments and a large protected luminal domain (27), as well as the cytosol-facing SNARE protein Bos1p (28) were monitored. Upon protease treatment, Erv46p shifted to a protease-protected species of the expected size (27), whereas Bos1p was fully digested (lanes 3 and 7). Addition of proteinase K and detergent caused digestion of all proteins examined (lanes 4 and 8). Collectively these observations indicate that the N and C termini of Erv26p are cytosolically exposed, consistent with the proposed topology model (Fig. 1B). Generation of a partially protected HA-Erv26 fragment in lane 3 likely arises through increased protease sensitivity of the C-terminal tail segment relative to the N terminus.

**Systematic Mutational Analysis of Erv26p**—In previous studies, we demonstrated that Erv26p was non-essential but required for efficient packaging of the vacuolar directed pro-ALP into ER-derived vesicles. To further investigate the mechanism by which Erv26p connects pro-ALP with the COPII coat, we systematically mutated specific amino acids in Erv26p, first targeting conserved residues outside of the transmembrane segments and then focusing on specific regions of the protein. Mutations were introduced into an ERV26-CEN plasmid, expressed in the erv26Δ background, and initially screened by immunoblot to determine Erv26p expression level and pro-ALP accumulation relative to wild type. Fig. 2 shows representative immunoblots of Erv26p stability and pro-ALP levels from a subset of mutations in the second and third loop domains. The complete results of the mutant analysis are provided in Table 2. Certain mutants, such as L3E and F71A/G72A/I73A severely destabilized Erv26p, but several other mutants, such as C75M and P120A, showed expression levels similar to wild-type Erv26p levels. Some of the stably expressed mutants also showed varying degrees of pro-ALP accumulation such as C75M (Fig. 2B) and F114A, D116A, P120A, and Q122A (Fig. 2A). To assess the kinetics of pro-ALP biogenesis, a subset of the pro-ALP-accumulating strains were further analyzed in pulse-chase experiments. Cells were pulsed with 35S-amino acids for 7 min to label newly synthesized proteins and chased with unlabeled amino acids to monitor ALP and carboxypeptidase Y maturation rates. As described previously (14), ALP maturation displayed a severe delay in erv26Δ strains, whereas carboxypeptidase Y matured at normal rates (Fig. 2C). The P120A and Q122A mutants displayed moderate delays in ALP maturation with half-times of ~10 and 14 min, respectively, compared with a wild-type rate of ~6 min. In addition, the C75M mutation decreased pro-ALP maturation (Fig. 2D) to a half-time of ~24 min. These reductions in pro-ALP processing indicated impaired Erv26p function; therefore we next examined specific mutants in cell-free...
assays to gain insight on the defects associated with single amino acid changes on Erv26p function.

Mutations in the Third Loop Domain Influence Interactions with Pro-ALP—Recent studies have indicated that the luminal region of pro-ALP as well as its proximity to the inner leaflet of the ER membrane is required for Erv26p-dependent ER export (49). To test whether amino acid residues in the third loop region of Erv26p are required for interactions between Erv26p and pro-ALP, we performed immunoprecipitation experiments with the P120A and Q122A mutants. Previous experiments demonstrated that wild-type Erv26p co-immunoprecipitated specifically with pro-ALP-HA (14). To assess co-immunoprecipitation of pro-ALP-HA with Erv26p mutants, semi-intact cells from pro-ALP-HA strains expressing wild-type Erv26p, Erv26-P120A, or Erv26-Q122A were solubilized in digitonin and immunoprecipitated with monoclonal Erv26p antiserum. In Fig. 3A, wild-type Erv26p co-immunoprecipitated pro-ALP-HA (22% of total); however, significantly less pro-ALP-HA was recovered with the P120A (0.08%) and Q122A (0.04%) mutants compared with wild type. Although more pro-ALP-HA was present in the total P120A and Q122A membrane extracts, association with these Erv26p mutants was clearly diminished. Erv41p, an ER/Golgi membrane protein, served as a negative control and confirmed specificity of the Erv26p association with pro-ALP-HA. The significant reduction in pro-ALP-HA co-immunoprecipitated with the P120A and Q122A loop domain mutants indicated that this region of Erv26p is important for cargo interactions. To assess whether mutations in the third loop domain affected other properties of Erv26p, we monitored packaging in COPII budding assays. Proteins that cycle between the ER and Golgi are packaged into COPII vesicles even in the absence of

**TABLE 2**

Analysis of Erv26p mutants

| Mutant  | ALP accumulation<sup>a</sup> | Erv26p stability<sup>b</sup> |
|---------|-------------------------------|-----------------------------|
| N-terminal tail |                               |                             |
| L2A/L3A/E4A/L5A | 26Δ                           | 26Δ                         |
| L2A/L3A | wt                            | −                           |
| L3E     | +                             | −                           |
| E4A     | −                             | −                           |
| L5A/I6A | +                             | −                           |
| L2A/L3A/E4A/L5A | 26Δ                           | 26Δ                         |
| L2A/L3A | wt                            | −                           |
| L3E     | +                             | −                           |
| E4A     | −                             | −                           |
| L5A/I6A | +                             | −                           |
| First loop domain |                               |                             |
| E32A    | wt                            | wt                          |
| E37A    | wt                            | wt                          |
| R42A    | wt                            | wt                          |
| E218A/E220A | +                             | −                           |
| F71A/S72A/I73A | +                             | −                           |
| S72A    | wt                            | wt                          |
| C75M    | +                             | −                           |
| Y76A/Y77A/Y78A/Y79A/Y80A | 26Δ                           | −                           |
| Y76A    | wt/Δ                          | wt                          |
| Y79A    | +                             | −                           |
| Q81A    | wt                            | wt                          |
| N82G    | wt                            | wt                          |
| L83N    | wt                            | −                           |
| K84A    | wt                            | −                           |
| Second loop domain |                               |                             |
| K67A/L68T | +                             | −                           |
| K73N    | wt                            | wt                          |
| F71A/S72A/I73A | +                             | −                           |
| S72A    | wt                            | wt                          |
| C75M    | +                             | −                           |
| Y76A/Y77A/Y78A/Y79A/Y80A | 26Δ                           | −                           |
| Y76A    | wt/Δ                          | wt                          |
| Y79A    | +                             | −                           |
| Q81A    | wt                            | wt                          |
| N82G    | wt                            | wt                          |
| L83N    | wt                            | −                           |
| K84A    | wt                            | −                           |
| Third loop domain |                               |                             |
| N106Δ/H107Δ | 26Δ                          | 26Δ                        |
| H107N   | wt                            | wt                          |
| K112Δ  | wt                            | wt                          |
| Y113Δ/F114Δ | +                             | −                           |
| F114Δ  | +                             | wt                          |
| D116Δ  | +                             | wt                          |
| E118Δ  | wt                            | wt                          |
| P120Δ  | +                             | wt                          |
| Q122Δ  | +                             | wt                          |
| K124Δ  | +                             | wt                          |
| C-terminal tail |                               |                             |
| E218A  | wt                            | wt                          |
| E218A/E220A | wt                          | −                           |
| D222A  | wt                            | wt                          |
| D222Δ/D224Δ | +                             | −                           |
| aa’205–211 to Ala | wt                            | wt                          |
| aa 198–204 to Ala | wt                            | wt                          |
| V199T/V200T | wt                            | wt                          |
| V200T  | wt                            | wt                          |
| ΔRLAV (aa 225–228) | wt                            | wt                          |
| Erv26C1-HA | +                             | wt                          |
| Erv26C2-HA | +                             | wt                          |
| Erv26-HA | +                             | wt                          |

<sup>a</sup> ALP key: +, pro-ALP accumulation approximately half the difference between Erv26 and erv26Δ; −, modest but obvious accumulation.

<sup>b</sup> Erv26p key: −, modest destabilization (75–90% of wt); −−, significant destabilization (less than 75%).

<sup>c</sup> aa, amino acids.
Molecular Dissection of Erv26p

A newly synthesized cargo (29), suggesting that binding of cargo to cargo receptors is not required for efficient packaging. The Erv26-P120A mutant was investigated in an in vitro COPII budding assay to determine whether the point mutation or a reduction in cargo interaction influenced packaging. Membranes from wild-type and Erv26-P120A strains were incubated in the presence or absence of purified COPII components to reconstitute budding from the ER. Vesicle-containing fractions were analyzed by immunoblot and compared with totals to monitor COPII-dependent packaging efficiency of specific proteins. Erv41p served as a positive control and was packaged in a COPII-dependent manner in both strains (Fig. 3B), whereas the ER-resident protein Sec61p was not packaged. Importantly the packaging efficiencies for wild-type Erv26p (13%) and Erv26-P120A (11%) were similar indicating that the P120A mutation does not have major influence on COPII recognition and packaging. Similarly the Erv26-Q122A mutant was also packaged at a similar efficiency (data not shown). These collective findings indicate that mutations in the third loop domain of Erv26p that resemble diacidic motifs: DFD (residues 222–224) and EIE (residues 218–220). However, when mutations were introduced altering the acidic residues in these sequences, the resulting mutants were apparently destabilized or had no significant increase in pro-ALP levels (Table 2). A sequence alignment of Erv26p with homologs across species showed little conservation in the C-terminal tail region. Other Erv26p homologs contain putative COPI sorting signals (-KKXX) at the extreme C terminus as do other yeast proteins that rely on this motif to cycle between the ER and Golgi compartments (27, 30, 31). However, mutation of the C-terminal four residues in Erv26p did not produce an apparent accumulation of pro-ALP when assessed under steady-state conditions (Table 2). To investigate the overall importance of the C-terminal cytosolic tail region for Erv26p function, a pair of truncation mutants were made in which most of the tail residues were deleted (Erv26ΔC1-HA, lacking residues 173–228; and Erv26ΔC2-HA, lacking residues 163–228). These two mutants were HA-tagged to facilitate detection because deletion of the C terminus removed the region used to generate the Erv26p-specific polyclonal antiserum. In addition, a full-length Erv26-HA tagged version was generated for comparison. As observed in Fig. 4A, deletion of the C-terminal tail had no significant effect on Erv26p expression level but resulted in a pro-ALP accumula-

FIGURE 3. Mutants in the third loop domain cause defects in pro-ALP interaction. A, semi-intact cell membranes containing ALP-HA and either wt (CYB2076), P120A (CYB2322), or Q122A (CYB2622) Erv26p were solubilized with digitonin, and Erv26p was immunoprecipitated with anti-Erv26 polyclonal antibodies. Gel lanes compare 5% of total membrane extracts (T) with immunoprecipitates (IP) in the presence (+) or absence (−) of anti-Erv26 polyclonal antibodies (Ab). Immunoblots were probed with antibodies against HA, Erv41p (integral membrane control), and Erv26p. Note the reduction in ALP-HA co-immunoprecipitated with Erv26-P120A and Erv26-Q122A compared with wild type. B, reconstituted COPII budding reactions from the indicated semi-intact cell membranes incubated with (+) or without (−) purified COPII proteins. Total (T) lanes represent one-tenth of the total reactions. Samples were immunoblotted with HA, Erv41p, and Erv26p antibodies. Note the similar packaging efficiencies of wild-type Erv26p and Erv26-P120A.

FIGURE 4. Mutants lacking the C-terminal tail accumulate pro-ALP. A, semi-intact cell membranes from wild type (CYB740), ΔC1-HA (CYB1480), ΔC2-HA (CYB2847), and full-length Erv26-HA (CYB2150) strains were assessed for content of the indicated proteins by immunoblot. B, wild type (CYB740), ΔC1-HA (CYB1480), ΔC2-HA (CYB2847), and full-length Erv26-HA (CYB2150) strains were analyzed in pulse-chase experiments as described under “Experimental Procedures.” Labels indicate the pro (p) and mature (m) forms of ALP as well as the ER (p1), Golgi (p2), and mature (m) forms of carboxypeptidase Y (CPY).
Molecular Dissection of Erv26p

Erv26p mutants that failed to interact efficiently with cargo were still efficiently incorporated into COPII vesicles (Fig. 3). To determine whether mutants deficient in COPII packaging show defects in cargo interaction, Erv26ΔC2-HA and Erv26-HA were analyzed in co-immunoprecipitation experiments. Microsomes were solubilized in 2% digitonin, and tagged proteins were recovered with monoclonal HA antibody. In these experiments the pro-ALP-HA fusion could not be used because HA tags were present on Erv26 constructs. Therefore, polyclonal ALP antibody was used in immunoblots. Brackets represent the position of peak Sec61p (ER marker), Och1p (Golgi marker), and Erv26 were determined by immunoblot.

FIGURE 5. The C-terminal tail sequence of Erv26p contains important sorting information. The subcellular distribution of Erv26p (CBY740), Erv26ΔC2-HA (CBY2847), and Erv26-HA (CBY2150) was monitored after separation of membranes on sucrose density gradients. Cell lysates were loaded on top of an 11-step sucrose gradient ranging from 18 to 60% sucrose and processed as described previously (14). Relative concentrations of Sec61p (ER marker), Och1p (Golgi marker), and Erv26 were determined by immunoblot. Brackets represent the position of peak Sec61p (ER)- and Och1p (Golgi)-containing fractions in each gradient. Sucrose concentrations across gradients were measured by refractometry.

The C-terminal tail sequence appeared to be important for proper Erv26p function and could play a role in both COPI- and COPII-directed movement. To determine how the C-terminal truncation influenced Erv26p trafficking, subcellular distribution was monitored after resolution of ER and Golgi membranes on sucrose density gradients. Whole cell membranes from strains expressing untagged Erv26p, Erv26-HA, and Erv26ΔC2-HA were fractionated on density gradients, and the fractionation patterns were assessed relative to ER (Sec61p) and Golgi (Och1p) marker proteins. As seen in Fig. 5, Golgi and ER marker proteins were resolved, and wild-type Erv26p co-fractionated with both ER and Golgi fractions (Fig. 5A) as observed previously (14). The Erv26ΔC2-HA was also detected in ER and Golgi fractions; however, the distribution was shifted more to the Golgi membrane fractions compared with wild-type Erv26p. Interestingly appending the HA tag onto the C terminus of full-length Erv26p strongly shifted Erv26-HA to Golgi-containing fractions (Fig. 5C). These results indicate that the C-terminal cytosolic tail of Erv26p is important for its normal steady-state distribution between ER and Golgi compartments. Both Erv26ΔC2-HA and Erv26-HA were shifted to Golgi membranes suggesting that these alterations interfered with normal COPI-dependent retrograde transport of Erv26p from the Golgi complex to the ER.

We next considered the possibility that Erv26-HA was more strongly shifted to Golgi membranes under steady-state conditions because COPII-dependent export from the ER was relatively efficient compared with COPI-dependent retrieval from Golgi membranes. In contrast, the Erv26ΔC2-HA protein, which produced a stronger pro-ALP accumulation phenotype, may be inefficiently packaged into both COPI and COPII carrier vesicles and therefore display an intermediate change in intracellular distribution. To directly assess COPII packaging efficiencies of Erv26ΔC2-HA and Erv26-HA, reconstituted budding assays were performed using ER-enriched microsomes prepared from the corresponding strains. As seen in Fig. 6A, wild-type Erv26p was packaged efficiently (28%), whereas Erv26ΔC2-HA was inefficiently (4.6%) incorporated into COPII vesicles. The level of Erv26-HA packaging was intermediate (14%); however, most cellular Erv26-HA was Golgi-localized (Fig. 5C), and this may reduce the apparent budding efficiency because microsomal membrane preparations contain some Golgi membranes. Regardless of this limitation, Erv26-HA was more efficiently packaged into COPII vesicles than was Erv26ΔC2-HA although the steady-state distribution of Erv26ΔC2-HA was greater in the ER than for Erv26-HA. This result is consistent with the proposal that deletion of the C-terminal tail interferes with both COPII and COPI sorting, whereas appending the HA tag interferes with normal COPII-dependent retrieval from the Golgi.

Erv26p mutants that failed to interact efficiently with cargo were still efficiently incorporated into COPII vesicles (Fig. 3). To determine whether mutants deficient in COPII packaging show defects in cargo interaction, Erv26ΔC2-HA and Erv26-HA were analyzed in co-immunoprecipitation experiments. Microsomes were solubilized in 2% digitonin, and tagged proteins were recovered with monoclonal HA antibody. In these experiments the pro-ALP-HA fusion could not be used because HA tags were present on Erv26 constructs. Therefore, polyclonal ALP antibody was used in immunoblots to detect any pro-ALP that was recovered with HA-tagged Erv26 proteins. As seen in Fig. 6B, pro-ALP was co-immunoprecipitated with both Erv26ΔC2-HA and Erv26-HA.
Molecular Dissection of Erv26p

over recovery of pro-ALP may be reduced with these HA-tagged versions of Erv26 because of partial Golgi mislocalization. However, specific associations between Erv26/H9004C2-HA and pro-ALP were detected suggesting that COPII interaction is not required for cargo binding.

Residues in the Second Loop Domain Are Important for Homodimerization—Several of the characterized protein sorting adaptors function as homo- or hetero-oligomers. For example, mammalian ERGIC-53 has been shown to form homodimers or homohexamers (32, 33), and Emp47p, the yeast homolog of ERGIC-53, assembles into homo-oligomers to augment incorporation into COPII vesicles (34). Although ERGIC-53 and Emp47p oligomerization is important for ER export, it is less clear how oligomerization influences cargo interaction (34, 35). Erv26p was shown previously to form homo-oligomers (36), and ALP is known to assemble into functional homodimers in yeast (37) as well as in other species (38). To probe the arrangement of the Erv26p homo-oligomer, chemical cross-linking followed by polyacrylamide gel analysis was used to determine the size of any cross-linked species. Wild-type microsomes were treated with increasing levels of the chemical cross-linker DSP, and proteins were resolved by non-reducing SDS-PAGE and immunoblotted with Erv26p antiserum (Fig. 7A). A single cross-linker-dependent product was detected that corresponded to approximately twice the size of monomeric Erv26p. This observation indicated that native Erv26p assembles into homodimers.

We next investigated whether the various Erv26p mutants influenced homodimerization. Native immunoprecipitation of Erv26-HA when expressed with untagged Erv26p point mutants provided a sensitive assay for homodimeric association. In Fig. 7B, immunoprecipitation of Erv26-HA from detergent-solubilized membranes recovered wild-type untagged Erv26p. However, the Erv26-I73N and Erv26-C75M mutants were not co-immunoprecipitated with Erv26-HA, suggesting that these mutations impaired Erv26p homodimerization.

Because of the effect that these putative cytosolic mutations had on stability of Erv26p homodimers, we next investigated whether the I73N and C75M mutations altered other interactions important for Erv26p function. Oligomerization of ER vesicle proteins has been shown to be required for efficient uptake into COPII vesicles (18, 34). To test packaging efficiencies of Erv26-I73N and Erv26-C75M compared with wild type, membranes were prepared from the appropriate strains and monitored in reconstituted COPII budding assays. As observed in Fig. 7C, the I73N and C75M versions were packaged as effi-

FIGURE 6. The C-terminal tail sequence of Erv26p contains an ER export signal. A, microsomes prepared from wild type (CBY740), Erv26ΔC2-HA (CBY2847), and Erv26-HA (CBY2150) strains were incubated in the presence (+) or absence (−) of purified COPII proteins, and budded vesicles were compared against total membranes (T) by immunoblot. Wild-type Erv26p was detected with anti-Erv26p polyclonal serum, and HA-tagged versions were detected with monoclonal HA antibody. B, microsomes from Erv26ΔC2-HA and Erv26-HA strains were solubilized with digitonin, and tagged proteins were immunoprecipitated (IP) with monoclonal HA antibody (Ab). Immunoprecipitation reactions and 5% of the total input material (T) were analyzed by immunoblot for pro-ALP (arrow indicates pro-ALP form), and Erv41p as negative control.

FIGURE 7. Native Erv26p exits as a homodimer. A, wild-type microsomes were treated with increasing concentrations of DSP (0.1, 0.2, and 0.4 mM) for 20 min at 20 °C before quenching with Tris-glycine. Samples were resolved on a non-reducing polyacrylamide gel and immunoblotted with Erv26p polyclonal antiserum. Arrows indicate positions of the Erv26p monomer and cross-linker-dependent Erv26p dimer. B, immunoprecipitation experiments used semi-intact cell membranes generated from strains expressing chromosomal Erv26-HA and a plasmid copy of wild-type Erv26p (CBY2305), Erv26-I73N (CBY2762), or Erv26-C75M (CBY2304). Membranes were solubilized in Triton X-100, and Erv26-HA was immunoprecipitated (IP) with HA antibody (Ab). Precipitated material and 5% of the total input (T) were analyzed by immunoblot of the indicated proteins. Note the reductions in Erv26-I73N and Erv26-C75M recovered compared with wild-type Erv26p. C, semi-intact cell membranes from the indicated strains were used in reconstituted COPII budding assays. Lanes include 10% of total reactions (T) and budded vesicles generated in the absence (−) or presence (+) of COPII proteins.

24056 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 284 • NUMBER 36 • SEPTEMBER 4, 2009
ciently as wild-type Erv26p. The results suggested that these mutations either do not severely disrupt Erv26p dimerization or that homodimer assembly is not required for COPII packaging. We favor the first interpretation because the C75M mutation did not inhibit production of the cross-linker-dependent Erv26p homodimer when microsomes containing this mutation were treated with DSP (data not shown). Instead we propose that the I73N and C75M mutations partially destabilize Erv26p homodimers that are then more susceptible to disassembly upon detergent solubilization of membrane complexes. Indeed mutations in this loop domain produced moderate pro-ALP accumulation phenotypes in vivo (Fig. 2) consistent with partial loss of function and not as severe as a null mutation as might be expected if mutants completely failed to dimerize.

We next investigated the ability of the weakened homodimer mutant, Erv26-C75M, to interact with its dimeric cargo, pro-ALP, in detergent-solubilized extracts. Membranes containing pro-ALP-HA and either wild type or Erv26-C75M were solubilized in 2% digitonin, and Erv26p was immunoprecipitated with polyclonal antiserum. Although pro-ALP-HA was co-immunoprecipitated with wild-type Erv26p, substantially less was recovered with the Erv26-C75M mutant even though the mutant membranes contained elevated levels of pro-ALP-HA (Fig. 8A). This result suggested that Erv26p dimerization may, in fact, be important for cargo interaction.

The COPII packaging efficiency and cargo interaction properties displayed by the Erv26-C75M mutant resembled those of the Erv26-P120A and Erv26-Q122A mutants. We therefore considered the possibility that cargo interaction may also influence stability of Erv26p homodimers. In the initial analysis of mutations residing in the third loop domain, homodimer instability had not been detected. However, to directly address this issue, we compared the levels of Erv26-P120A and Erv26-C75M that dimerized with HA-tagged protein in detergent extracts through co-immunoprecipitation with monoclonal HA antibodies. In Fig. 8B, we observed that Erv26-P120A co-immunoprecipitated with Erv26-HA, whereas Erv26-C75M was not recovered in association with the tagged wild-type protein. In summary, mutations in the second loop domain interfere with homodimerization and cargo interaction, whereas mutations in the third loop produce distinct defects in cargo interaction and not in homodimer stability.

**Ktr3p Is Dependent on Erv26p for ER Export**—When Erv26p immunoprecipitates were examined by protein stain, we consistently observed an additional polypeptide of ~43 kDa (data not shown). An Erv26p-associated protein of similar size was identified as Ktr3p, a Golgi-localized Type II transmembrane mannose-6-phosphate receptor (36). Moreover Ktr3p was shown to mislocalize to ER membranes in erv26Δ strains (36). Based on this evidence, we tested whether Ktr3p packaging into COPII vesicles was Erv26p-dependent using inhibitory anti-Erv26p antibodies (14). COPII budding reactions were performed with microsomes from a Ktr3-HA strain in the presence of anti-Erv26p antiserum or preimmune serum, and packaging was analyzed by immunoblot. As seen in Fig. 9A, Erv26p was packaged efficiently in the presence of preimmune serum but was inhibited by addition of the Erv26p antiserum. Likewise COPII packaging of Ktr3-HA was reduced by 61% in the presence of inhibitory Erv26p antibodies. As a control for specificity of the inhibition, the ER vesicle protein Erv41p continued to be packaged efficiently in the presence of Erv26p antibodies. These results indicate that efficient uptake of Ktr3-HA into COPII vesicles depends on Erv26p and support a model in which Erv26p acts as a sorting receptor for multiple cargo.

Mutations in the third loop domain of Erv26p interfered with pro-ALP interaction. To determine whether these mutations similarly influenced interactions with Ktr3-HA, co-immunoprecipitation experiments were performed from strains expressing Ktr3-HA and wild-type Erv26p or the Erv26-P120A mutant. Membranes were solubilized in digitonin, and Ktr3-HA was immunoprecipitated with monoclonal HA antibody. As seen in Fig. 9B, Ktr3-HA was efficiently recovered, and wild-type Erv26p was co-immunoprecipitated. However, Erv26-P120A was not detected in Ktr3-HA immunoprecipitates, indicating that this mutant does not interact efficiently with Ktr3-HA as was observed for pro-ALP-HA. Taken together, the packaging and co-immunoprecipitation data indicate that Ktr3p is a second Type II transmembrane cargo protein that relies on Erv26p for ER export and probably interacts with this sorting receptor through a conserved mechanism.

**DISCUSSION**

Initial reports indicated that Svp26p/Erv26p traffics in the early secretory pathway and directs pro-ALP into COPII vesicles for efficient export from the ER (14, 36). Although these studies established Erv26p-dependent transport of pro-ALP, the molecular mechanism by which Erv26p connects cargo to
the COPII coat remained unclear. Here we report a site-directed mutational analysis of Erv26p to refine the molecular model for Erv26p-dependent export. Stably expressed Erv26p mutants that produced delayed pro-ALP transport were characterized to determine underlying defects. Mutations in the third loop domain of Erv26p specifically influenced cargo interactions, whereas mutations in the C-terminal cytosolic tail reduced COPII packaging and altered subcellular localization. Importantly these findings demonstrate that Erv26p cargo binding and coat recognition are independent because mutants were identified that influenced one activity and not the other. In contrast, specific mutations in the second loop domain affected Erv26p homodimerization and cargo binding suggesting that these activities may be coupled. Finally we showed that the cargo protein Ktr3p also depends on Erv26p for efficient ER export indicating a more general role for this cargo receptor in transport of Type II transmembrane proteins.

Molecular Dissection of Erv26p

FIGURE 9. Ktr3p depends on Erv26p for COPII packaging. A, in vitro budding reactions from Ktr3-HA membranes (CBY2548) were incubated with (+) or without (−) COPII proteins and with preimmune serum (+P) or polyclonal Erv26 antibody (+α26). Total [3H]lanes represent one-tenth of the total budding reactions. B, microsomes from HA-tagged Ktr3p strains expressing wild-type Erv26p (CBY2548) or Erv26-P120A (CBY2767) were solubilized with digitonin, and tagged proteins were immunoprecipitated with anti-HA monoclonal antibodies. After immunoblot of the indicated proteins, lanes compare 5% of total membrane extracts (T) with immunoprecipitated material (IP) in the presence (+) or absence (−) of anti-HA antibodies (Ab).

these mutations could generally distort the Erv26p structure; however, we showed that amino acid changes in the third loop region neither destabilized Erv26p nor altered normal COPII recognition. Regardless a structural analysis of Erv26p will be necessary to unambiguously confirm our proposal. In instances where structural information is available for sorting receptors, the ERGIC-53 protein contains a luminal L-type lectin domain that binds specific cargo in part through carbohydrate recognition (39, 40). We note that pro-ALP contains N-linked core oligosaccharides; however, mutation to remove these sites and transport rates in the presence of tunicamycin (41) indicate that carbohydrate recognition is not required for Erv26p-dependent sorting. Therefore we speculate that Erv26p binds pro-ALP and other cargo through recognition of a protein structural element in the folded cargo.

Our mutant analysis revealed that the cytoplasmic C-terminal tail sequence was required for normal trafficking and function of Erv26p. Deletion of entire C-terminal tail (Erv26ΔC2-HA) produced a severe delay in pro-ALP transport, shifted the subcellular distribution of Erv26p to Golgi membranes, and decreased COPII incorporation into budded vesicles. Appending the HA tag to the C terminus of full-length Erv26p also caused a shift in localization to Golgi membranes, although Erv26-HA displayed only mild reductions in pro-ALP transport and recognition by the COPII budding machinery. Based on these results, we conclude that the C-terminal tail of Erv26p contains COPII-specific sorting signals that allow the protein to cycle between ER and Golgi compartments in accord with other shuttling cargo receptors (31, 42, 43). We speculate that removal of both transport signals in the tail deletion mutant (Erv26ΔC2-HA) produces a protein that is inefficiently packaged into COPII vesicles but ultimately traffics to the Golgi complex in a slower “bulk flow” manner. Lack of a COPII retrieval signal in the tail deletion protein would be expected to shift the steady-state distribution of Erv26ΔC2-HA to Golgi membranes as was observed. In the case of the Erv26-HA, the steady-state distribution was also shifted to Golgi membranes possibly because of partial interference with a C-terminal COPI signal. However, the mild accumulation of pro-ALP and efficient COPII packaging observed suggests that Erv26-HA cycles at near wild-type rates.

Further investigation of the C-terminal tail of Erv26p may reveal specific signals for COPI and COPII interaction. The Erv26p tail sequence contains potential COPII sorting signals including diacidic (4) and hydrophobic/aromatic motifs (27, 31, 42) that could bind directly to sites in the Sec24p subunit of COPII (1). However, mutational analysis of these stretches produced wild-type phenotypes or destabilized Erv26p protein (Table 2). It is possible that redundant or overlapping sorting signals reside in the Erv26p tail sequence and precluded simple dissection. Regardless a structural analysis of Erv26p will be necessary to unambiguously confirm our proposal. In instances where structural information is available for sorting receptors, the ERGIC-53 protein contains a luminal L-type lectin domain that binds specific cargo in part through carbohydrate recognition (39, 40). We note that pro-ALP contains N-linked core oligosaccharides; however, mutation to remove these sites and transport rates in the presence of tunicamycin (41) indicate that carbohydrate recognition is not required for Erv26p-dependent sorting. Therefore we speculate that Erv26p binds pro-ALP and other cargo through recognition of a protein structural element in the folded cargo.

Chemical cross-linking and immunoprecipitation experiments indicated that native Erv26p exists as a homodimer.
Mutations in the second loop domain (I73N and C75M) destabilized Erv26p dimer associations, reduced Erv26p interaction with cargo, and produced delays in pro-ALP transport in vivo. Other ER-Golgi protein sorting adaptors are known to oligomerize. ERGIC-53 forms homodimers and homohexamers (33), which are required for ER export of ERGIC-53 (9, 42). In addition, Emp47p assembles into homo-oligomers and hetero-oligomers with Emp46p for efficient uptake into COPII vesicles (34). There are now many examples in which oligomeric assembly of membrane proteins is needed for COPII-dependent recognition and export (1). However, it is less clear how oligomeric arrangements could influence cargo binding to sorting receptors. In the case of Erv26p, the I73N and C75M mutations destabilized Erv26p homodimers in detergent extracts, whereas COPII-dependent export and overall trafficking of the mutants appeared unchanged. These results may be explained if the point mutations weakened contacts at an Erv26p dimer interface or introduced conformational changes in the overall structure but not to an extent that prevented dimer assembly. Such mutations could allow Erv26p trafficking to proceed normally but interfere with the geometry of binding interactions between dimeric pro-ALP and dimeric Erv26p. Further biochemical and structural analyses will be needed to determine these molecular arrangements, although the availability of these semifunctional Erv26p mutants should facilitate such studies.

Binding of nascent secretory cargo to sorting receptors is thought to be regulated in a manner that promotes cargo association at ER exit sites and then releases bound cargo in post-ER or early Golgi compartments. Regulatory mechanisms may incorporate pH or Ca$^{2+}$ gradients that exist between lumenal compartments of the early secretory pathway (44, 45). Indeed biochemical studies on interactions between the ERGIC-53 cargo receptor and high mannose glycoproteins indicated that binding affinity is influenced by both pH and Ca$^{2+}$ concentrations in correlation with intracellular gradients (35, 46). Alternatively clustering of cargo receptors by COPII coat subunits at ER exit sites could increase receptor affinity for cargo and then reverse ligand binding upon vesicle uncoating. In these models, the oligomeric arrangement of Erv26p and other known cargo receptors could provide a means for inducing conformational changes that favor cargo binding in the ER and then shift to an unbound closed state in post-ER compartments as observed for other types of multimeric receptor proteins (47, 48). In the case of Erv26p, our studies show that deletion of the C-terminal tail sequence uncoupled coat recognition from cargo binding suggesting that COPII clustering of cargo receptors does not regulate cargo interaction. Instead these results support a model where shifts in the lumenal environment change cargo affinity possibly through inducing conformation changes in the Erv26p homodimer. Binding studies with isolated Erv26p and pro-ALP combined with in vitro assays of ER-Golgi transport under conditions that vary lumenal environments should allow us to directly test this model.

Acknowledgments—We thank members of the Barlowe laboratory for valuable discussions and Julia Dancourt for critical reading of the manuscript.

REFERENCES

1. Lee, M. C., Miller, E. A., Goldberg, J., Orci, L., and Schekman, R. (2004) Annu. Rev. Cell Dev. Biol. 20, 87–123
2. Glirkan, C., Stagg, S. M., Lapointe, P., and Balch, W. E. (2006) Nat. Rev. Mol. Cell Biol. 7, 727–738
3. Kappeler, F., Klupfenstein, D. R., Fuguet, M., Paccoud, J. P., and Hauri, H. P. (1997) J. Biol. Chem. 272, 31801–31808
4. Nishimura, N., and Balch, W. E. (1997) Science 277, 556–558
5. Mossoleva, E., Bickford, L. C., and Goldberg, J. I. (2003) Cell 114, 483–495
6. Miller, E. A., Belharz, T. H., Malkus, P. N., Lee, M. C., Hamamoto, S., Orci, L., and Schekman, R. (2003) Cell 114, 497–509
7. Sato, K., and Nakano, A. (2007) FEBS Lett. 581, 2076–2082
8. Nichols, W. C., Seligsohn, U., Zivelin, A., Terry, V. H., Hertel, C. E., Wheatley, M. A., Moussalli, M. I., Hauri, H. P., Ciavarella, N., Kaufman, R. J., and Ginsburg, D. (1998) Cell 93, 61–70
9. Appenzeller, C., Andersson, H., Kappeler, F., and Hauri, H. P. (1999) Nat Cell Biol. 1, 330–334
10. Nyfeler, B., Reiterer, V., Wendeler, M. W., Stefan, E., Zhang, B., Michnick, S. W., and Hauri, H. P. (2008) J. Cell Biol. 180, 705–712
11. Hauri, H. P., Kappeler, F., Andersson, H., and Appenzeller, C. (2000) J. Cell Sci. 113, 587–596
12. Otte, S., Belden, W. J., Heidtman, M., Liu, J., Jensen, O. N., and Barlowe, C. (2001) J. Cell Biol. 152, 503–518
13. Belden, W. J., and Barlowe, C. (2001) Science 294, 1528–1531
14. Bue, C. A., Bentivoglio, C. M., and Barlowe, C. (2006) Mol. Biol. Cell 17, 4780–4789
15. Sherman, F. (1991) Methods Enzymol. 194, 3–21
16. Ausubel, R. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) Current Protocols in Molecular Biology, pp. 3.0.1–3.14.3, John Wiley & Sons, Inc., New York
17. Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachter, A., Philipp, P., and Pringle, J. R. (1998) Yeast 14, 953–961
18. Belden, W. J., and Barlowe, C. (1996) J. Biol. Chem. 271, 26939–26946
19. Stirling, C. J., Rothblatt, J., Hosobuchi, M., Deshaies, R., and Schekman, R. (1992) Mol. Biol. Cell 3, 129–142
20. Liu, Y., and Barlowe, C. (2002) Mol. Biol. Cell 13, 3314–3324
21. Haas, A., Schegtmann, D., Lazar, T., Gallwitz, D., and Wickner, W. (1995) EMBO J. 14, 5258–5270
22. Baker, D., Hicke, L., Rexach, M., Schleyer, M., and Schekman, R. (1988) Cell 54, 335–344
23. Wuesthube, L. J., and Schekman, R. W. (1992) Methods Enzymol. 199, 124–136
24. Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M. F., Ravazzola, M., Amherdt, M., and Schekman, R. (1994) Cell 77, 895–907
25. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. (2001) J. Mol. Biol. 305, 567–580
26. von Heijne, G. (1992) J. Mol. Biol. 225, 487–494
27. Otte, S., and Barlowe, C. (2002) EMBO J. 21, 6095–6104
28. Newman, A. P., Groesch, M. E., and Ferro-Novick, S. (1992) EMBO J. 11, 3609–3617
29. Yeung, T., Barlowe, C., and Schekman, R. (1995) J. Biol. Chem. 270, 30567–30570
30. Schroeder, S., Schimmel, F., Singer-Krueger, B., and Riezman, H. (1995) J. Biol. Cell 131, 895–912
31. Sato, K., and Nakano, A. (2002) Mol. Biol. Cell 13, 2518–2532
32. Schweizer, A., Fransen, J. A., Bachi, T., Ginsel, L., and Hauri, H. P. (1988) J. Biol. Cell 107, 1643–1653
33. Neve, E. P., Lahtinen, U., and Pettersson, R. F. (2005) J. Mol. Biol. 354, 556–568
34. Sato, K., and Nakano, A. (2003) Mol. Biol. Cell 14, 3055–3063
35. Appenzeller-Herzog, C., Roche, A. C., Nufer, O., and Hauri, H. P. (2004) J. Biol. Chem. 279, 12943–12950
36. Inadome, H., Noda, Y., Adachi, H., and Yoda, K. (2005) Mol. Cell Biol. 25, 7696–7710
37. Onishi, H. R., Tkacz, J. S., and Lampen, J. O. (1979) J. Biol. Chem. 254, 11943–11952
38. Boulanger, R. R., Jr., and Kantrowitz, E. R. (2003) J. Biol. Chem. 278, 23497–23501
39. Itin, C., Roche, A. C., Monsigny, M., and Hauri, H. P. (1996) Mol. Biol. Cell 7, 483–493
40. Velloso, L. M., Svensson, K., Schneider, G., Pettersson, R. F., and Lindqvist, Y. (2002) J. Biol. Chem. 277, 15979–15984
41. Clark, D. W., Tkacz, J. S., and Lampen, J. O. (1982) J. Bacteriol. 152, 865–873
42. Nufer, O., Guldbrandsen, S., Degen, M., Kappeler, F., Paccaud, J. P., Tani, K., and Hauri, H. P. (2002) J. Cell Sci. 115, 619–628
43. Belden, W. J., and Barlowe, C. (2001) J. Biol. Chem. 276, 43040–43048
44. Paroutis, P., Touret, N., and Grinstein, S. (2004) Physiology 19, 207–215
45. Pezzati, R., Bossi, M., Podini, P., Meldolesi, J., and Grohovaz, F. (1997) Mol. Biol. Cell 8, 1501–1512
46. Kawasaki, N., Ichikawa, Y., Matsuo, I., Totani, K., Matsumoto, N., Ito, Y., and Yamamoto, K. (2008) Blood 111, 1972–1979
47. Olson, L. J., Hindsgaul, O., Dahms, N. M., and Kim, J. J. (2008) J. Biol. Chem. 283, 10124–10134
48. Ward, C. W., Lawrence, M. C., Streltsov, V. A., Adams, T. E., and McKern, N. M. (2007) Trends Biochem. Sci. 32, 129–137
49. Dancourt, J., and Barlowe, C. (2009) Traffic 10, 1006–1018