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Erv26p Directs Pro-Alkaline Phosphatase into Endoplasmic Reticulum–derived Coat Protein Complex II Transport Vesicles[D]

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Secretory proteins are exported from the endoplasmic reticulum (ER) in transport vesicles formed by the coat protein complex II (COPII). We detected Erv26p as an integral membrane protein that was efficiently packaged into COPII vesicles and cycled between the ER and Golgi compartments. The *erv26Δ* mutant displayed a selective secretory defect in which the pro-form of vacuolar alkaline phosphatase (pro-ALP) accumulated in the ER, whereas other secretory proteins were transported at wild-type rates. In vitro budding experiments demonstrated that Erv26p was directly required for packaging of pro-ALP into COPII vesicles. Moreover, Erv26p was detected in a specific complex with pro-ALP when immunoprecipitated from detergent-solublized ER membranes. Based on these observations, we propose that Erv26p serves as a transmembrane adaptor to link specific secretory cargo to the COPII coat. Because ALP is a type II integral membrane protein in yeast, these findings imply that an additional class of secretory cargo relies on adaptor proteins for efficient export from the ER.

INTRODUCTION

The eukaryotic secretory pathway transports a remarkable variety of cargo proteins to their proper cellular location. Several lines of evidence indicate that targeting information encoded within a secretory protein is recognized by the intracellular transport machinery to direct localization. Cytosolic coat protein complexes play an important role in deciphering targeting information and act in the selection of secretory proteins into appropriate transport intermediates (Bonifacino and Glick, 2004). However, the sorting signals and mechanisms by which coat complexes accommodate such diversity in secretory cargo remain poorly understood.

Protein transport from the endoplasmic reticulum (ER) relies on coat protein complex II (COPII), which selects fully folded secretory cargo into ER-derived transport intermediates. COPII coats consist of the small GTPase Sar1p, the Sec23/24 complex, and the Sec13/31 complex (Barlowe *et al*., 1994). Biochemical and structural studies have demonstrated that the Sec24p subunit of this coat complex contains multiple cargo recognition sites and binds specific sorting signals displayed on the cytosolic regions of secretory proteins (Miller *et al*., 2003; Mossessova *et al*., 2003). Diacidic sequences and other polypeptide sorting signals have been identified in cargo proteins that interact with amino acid residues within defined Sec24p cargo recognition sites. Current models envisage that the cargo recognition and binding by Sec24p is stabilized through assembly of prebudding complexes consisting of Sec23/24 and Sar1p-GTP bound to secretory cargo on the ER membrane surface. These prebudding complexes are then incorporated into an outer layer Sec13/31 scaffold structure that deforms the membrane and produces COPII-coated vesicles (Lee *et al*., 2004; Stagg *et al*., 2006).

In addition to the COPII coat proteins, cargo-specific accessory factors are required to accommodate the variety of secretory cargo exported from the ER in COPII vesicles. For example, efficient export of certain soluble secretory proteins from the ER depends on transmembrane receptor-like proteins. ERGIC53, an integral membrane protein that cycles between the ER and Golgi complex is required for ER export of secreted blood coagulation factors and procathepsin Z in mammalian cells (Appenzeller *et al*., 1999; Appenzeller-Herzog *et al*., 2005). In yeast, transmembrane Erv29p is required for efficient ER export of the soluble secretory proteins pro-α-factor and carboxypeptidase Y (CPY) (Belden and Barlowe, 2001). In cells that lack ERGIC53 or Erv29p activity, subsets of the secretory cargo accumulate in the ER, whereas COPII-dependent transport of most secretory proteins proceeds normally. These findings indicate that adaptor molecules are needed to link specific secretory proteins to the COPII coat. Although these types of cargo receptors are packaged into COPII vesicles and actively cycle between ER–Golgi compartments, other cargo-specific accessory factors seem to be required for assembly and/or insertion of cargo into ER-derived vesicles but do not accompany the cargo into vesicles. For example, ER-localized NinaA in *Drosophila melanogaster* (Baker *et al*., 1994) and Shr3p in yeast (Kuehn *et al*., 1996) are in some way required to incorporate specific cargo into ER-derived vesicles. Further studies are needed to fully understand the mechanisms that underlie COPII-dependent export of proteins from the ER.

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Abbreviations used: ALP, alkaline phosphatase; CPY, carboxypeptidase Y; COPII, coat protein complex II; gpαf, glycopro-α-factor; UPR, unfolded protein response; UPRE, UPR response element.

Given the diversity in secretory cargo exported from the ER, we hypothesize that additional cargo-specific transport factors are required to mediate efficient ER export. Our approach has been to investigate the function of abundant ER vesicle proteins (Ervs) that are enriched in purified COPII vesicle preparations (Otte *et al*., 2001). Several of the Erv proteins are involved in cargo sorting and when mutated they produce selective protein sorting defects (Schimmoller *et al*., 1995; Belden and Barlowe, 1996; Powers and Barlowe, 1998; Belden and Barlowe, 2001). In this report, we identify and characterize a new cargo-specific ER export factor, Erv26p. Erv26p is packaged into COPII vesicles, cycles between the ER and Golgi compartments and is required for efficient ER export of vacuolar localized alkaline phosphatase (ALP). Other secretory proteins we examined in the *erv26*Δ strain were transported from the ER at wild-type rates. Interestingly, the secretory protein that accumulates in an *erv26*Δ mutant, pro-ALP, is a type II integral membrane protein. These findings suggest that an additional class of secretory cargo rely on adaptor proteins for efficient coupling to the COPII coat and export from the ER.

MATERIALS AND METHODS

Yeast Strains and Media

Strains used in this study are listed in Table 1. Cells were grown in YPD rich medium (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose) or YMD minimal medium (0.67% nitrogen base without amino acids, 2% dextrose) with appropriate supplements, at 30°C. Standard yeast (Sherman, 1991) and bacteria (Ausubel *et al*., 1987) molecular genetic methods were used.

Plasmid Construction

The *ERV26* gene including 300 base pairs upstream and downstream of the predicted open reading frame was amplified by polymerase chain reaction (PCR) from genomic DNA prepared from strain FY834 (Winston *et al*., 1995) by using primers Erv26fl-F (GAAGATCTGA ACGTTTGGGA TGACGTATTG) and Erv26fl-R (GGGGTACCTC TACTGGGCCT GACTGG). BglII and EcoRI restriction sites were used to subclone this PCR product into pRS316 (Sikorski and Hieter, 1989) and pRS426 (Christianson *et al*., 1992). The *PHO8* multicopy expression plasmid for overexpression of ALP was constructed by PCR amplification of FY834 genomic DNA by using the primers Pho8EcoR1-F (GGAATTCCCC CCTCGTAAGG CGCGTCT) and Pho8BamHI-R (CGGGATCCCG CAATCTG-GAC TGAATGGC) that anneal 240 base pairs upstream and downstream, respectively, of the ALP open reading frame. EcoRI and BamHI sites were used to subclone this PCR product into pRS423 (Christianson *et al*., 1992). Plasmid constructs were sequenced to confirm correct synthesis.

Strain Construction

The *erv26::KAN* strain was obtained from the Research Genetics deletion strain collection (Winzeler *et al*., 1999). The ALP-hemagglutinin (HA)–tagged strain was constructed by targeting the *PHO8* gene with the PCR product generated from pFA6a-3HA-His3MX6 (Longtine *et al*., 1998) by using primers PHO8-HA.F2 (GTGAAGTACA ACATTATGAC GAATACTACC ATGAGTT-GAC CAACCGGATC CCCGGGTTAA TTA) and PHO8-HA.R1 (CGTATTA-AAT AATATGTGAA AAAAGAGGGA GAGTTAGATA GGAGCATAGG CCACTAGTGG).

Antibodies and Immunoblotting

Anti-Erv26p polyclonal antibodies were raised against a glutathione *S*-transferase (GST)-Erv26p fusion protein containing amino acid residues 159–228 of the Erv26p predicted open reading frame. Primers SV26-B (CGGGATCCTC AGCTGGTGAT TATGTG) and SV26-E (CGGAATTCCT AAACAGCCAA TCTATCAAAG) were used to PCR amplify a segment of *ERV26*. The resulting PCR product was treated with BamHI and EcoRI and inserted into BamHI/EcoRI-digested pGEX-2T (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). This GST-Erv26p fusion was expressed as a 30-kDa protein in DH5α cells and was contained in soluble extracts after lysis in a French Press. Fusion protein was purified on a glutathione-agarose column according to the manufacturer's specifications (GE Healthcare) and used to produce antiserum in rabbits (Covance, Denver, PA). The anti-Erv26p serum was typically used at a 1:1000 dilution for immunoblotting. Antibodies directed against Sec23p (Hicke and Schekman, 1989), Erv25p (Belden and Barlowe, 1996), Sec61p (Stirling *et al*., 1992), Erv46p and Sec22p (Liu and Barlowe, 2002), Gas1p (Fankhauser and Conzelmann, 1991), CPY (Rothblatt *et al*., 1989), ALP (Haas *et al*., 1995), Vam3p (Wang *et al*., 2003), and Och1p, Erv41p, and Erv46p (Otte *et al*., 2001) have been described previously. A monoclonal HA antibody (HA.7) was obtained from Sigma-Aldrich (St. Louis, MO). Immunoblots were developed using the enhanced chemiluminescence method (GE Healthcare), and densitometric analysis was performed on scanned images of immunoblots using the ImageJ program (National Institutes of Health, Bethesda, MD).

In Vitro Budding Assays

Large-scale in vitro budding was performed on microsomes in the absence or presence of purified COPII components (Barlowe *et al*., 1994). A 10-μl portion of the total reaction and 200 μl of supernatant containing vesicles were centrifuged at 60,000 rpm (TLA100.3 rotor; Beckman Coulter, Fullerton, CA) to pellet membranes. Membrane pellets were resuspended in SDS-PAGE sample buffer, and 8- to 10-μl aliquots were loaded on either 12.5 or 8% polyacrylamide gels for immunoblots. Where indicated, preimmune or anti-Erv26p antibodies were added to membranes and incubated on ice for 15 min before the addition of budding factors. COPII budding reactions were performed at 25°C.

Subcellular Fractionation

To characterize the membrane association of Erv26p, wild-type microsomes were suspended in buffer 88 (20 mM HEPES, pH 7.5, 250 mM sorbitol, 150 mM KOAc, and 5 mM MgOAc), in buffer 88 containing 0.5 M NaCl, in buffer 88 containing 0.1% Triton X-100, or in a carbonate buffer (0.1 M Na_2CO_3, pH 11). Samples were mixed and incubated on ice for 10 min and were then spun at 60,000 rpm (TLA 100.3 rotor; Beckman Coulter) for 12 min at 4°C. Equivalent amounts of the total, high-speed supernatant, and high-speed pellet fractions were diluted in SDS-PAGE sample buffer and resolved on a 12.5% polyacrylamide gel.

Subcellular fractionation was performed as described in Powers and Barlowe (1998) with minor modification as follows. Strains were grown to log phase and spheroplasted by lyticase treatment (Baker *et al*., 1988). Spheroplasts were resuspended with a sucrose solution (10 mM HEPES, pH 7.5, 1 mM EDTA, 12.5% sucrose, and 0.5 mM phenylmethylsulfonyl fluoride) and subjected to Dounce homogenization. The homogenate was spun at low speed (3500 rpm in an SS34 for 4 min), and the resulting low-speed supernatant fraction was loaded on an 11-step sucrose gradient (ranging from 18 to 60% sucrose in 10 mM HEPES pH 7.5 and 1 mM $MgCl_2$). Sucrose step gradients were centrifuged for either 3 h at 36,000 rpm or 2.5 h at 35,000 rpm

Table 1. Yeast strains used in this study

Strain	Genotype	Source
CBY740	*MATα his3 leu2 lys2 ura3*	BY4742 Research Genetics (Huntsville, AL)
CBY1480	*MATα his3 leu2 lys2 ura3 erv26ΔKAN^R*	16420 Research Genetics
CBY1912	CBY1480 with pRS316-*ERV26*	This study
CBY1913	CBY1480 with pRS426-*ERV26*	This study
CBY2076	*MATα his3 leu2 lys2 ura3 PHO8-3XHA-HIS3MX6*	This study
CBY2077	*MATα his3 leu2 lys2 ura3 erv26ΔKAN^R PHO8-3XHA-HIS3MX6*	This study
CBY2140	CBY740 with pRS423-*PHO8*	This study
CBY2194	CBY2140 with pRS426-*ERV26*	This study
RSY248	*MATα his4-619*	Kaiser and Schekman (1990)
RSY263	*MATα ura3-52 sec12-4*	Kaiser and Schekman (1990)

(SW40 rotor; Beckman Coulter). Fractions of 750 μl were taken from top to bottom and diluted 2:1 in SDS-PAGE sample buffer, and proteins were resolved on either a 12.5 or 8% polyacrylamide gel. Fractions were blotted for Erv26p, ALP, Och1p (Golgi marker), Sec61p (ER marker), Vam3p (vacuole marker), and Erv46p (ER vesicle protein).

In Vivo Labeling

Pulse-chase experiments were performed as described previously (Belden and Barlowe, 1996) with minor modifications. Wild-type (CBY740) and *erv26*Δ (CBY1480) cells were grown in media with reduced sulfate to an OD_{600} of 0.4. Cells were harvested, washed, and resuspended at an OD_{600} of 3.0 in minimal media without sulfate. After preincubation at 30°C for 5 min, cultures were pulsed with [^{35}S]Promix (GE Healthcare) at 25 μCi/OD_{600} cells. The chase phase was initiated after 7 min by the addition of excess unlabeled methionine and cysteine. Cells were collected at 0-, 5-, 10-, or 20-min time points, and extracts were prepared as described previously (Belden and Barlowe, 1996). Gas1p, CPY, and ALP were immunoprecipitated from common extracts; resolved on 10% polyacrylamide gels; and labeled species visualized by fluorography.

Fluorescence Microscopy and Green Fluorescent Protein (GFP) Fluorescence-activated Cell Sorting (FACS) Analysis

For indirect immunofluorescence, yeast cells were prepared and mounted as described previously (Powers and Barlowe, 1998). Immunofluorescent images were collected with a Hamamatsu (Bridgewater, NJ) Orca II cooled charged-coupled device camera mounted on a Zeiss Axioplan 2 microscope (Carl Zeiss, Thornwood, NY), using OPENLAB software (Improvision, Lexington, MA).

The GFP-based unfolded protein response (UPR) activation assay was performed as described previously (Travers *et al*., 2000). Flow cytometry was performed on a Becton Dickinson FACScan instrument. Single integration of the pUPRE-GFP-URA3 plasmid was confirmed in individual strains by measuring the fully induced UPR after treatment with 2 mM dithiothreitol for 2.5 h.

Immunoprecipitation of ALP-HA/Erv26p Complexes

Immunoprecipitation experiments were performed according to Heidtman *et al*. (2005) with the following modifications. Wild-type microsomes (220 μg of total membrane protein in 225 μl) containing ALP or HA-tagged ALP were solubilized in an equal volume of buffer 88, pH 8.0, containing 2% digitonin (B88-8/2.0% digitonin) in the presence of 1 mM phenylmethylsulfonyl fluoride and 5 mM EDTA. After centrifugation at 14,000 rpm for 5 min at room temperature to pellet unsolubilized material, ~400 μl of the supernatant fractions were recovered. Then, 10% of the solubilized material was saved as a total sample, and the remainder was diluted with 2 volumes of B88-8/0.05% digitonin. To immunoprecipitate ALP-HA, 0.25 μg of the monoclonal HA antibody, and 40 μl of 20% protein A-Sepharose beads were added. After binding for 1.5 h at 4°C, immunocomplexes bound to Sepharose beads were washed four times with B88-8/0.05% digitonin. Bound proteins were eluted from the beads by adding 40 μl of SDS-PAGE sample buffer followed by incubation at 75°C for 4 min. Total and immunoprecipitated (IP) samples were then resolved on polyacrylamide gels and analyzed by immunoblot.

RESULTS

Erv26p Is a Component of COPII-coated Vesicles

In previous studies, we identified several of the abundant proteins contained on uncoated COPII vesicles by mass spectrometry, including Erv14p, Erv25p, Erv29p, Erv41p, and Erv46p (Belden and Barlowe, 1996; Powers and Barlowe, 1998; Otte *et al*., 2001). On further analysis of the protein species migrating with Erv25p, we detected polypeptides derived from the putative open reading frame YHR181w. The conceptual translation of YHR181w produces a 26-kDa tetraspanning membrane protein that is widely conserved across species but of unknown function. Whereas YHR181w does not contain characterized sequence motifs that provide insight into molecular function, homologues in *D. melanogaster* and in mammalian species terminate in the sequences -KKXX or -QKXX. These dilysine-based sorting signals are likely to be recognized by the COPI coat complex and act in retrograde transport between early compartments of the secretory pathway. We termed YHR181w Erv26p, for ER vesicle protein of 26 kDa, and explored its function in ER–Golgi transport. During the course of our studies, Inadome and colleagues also identified YHR181w as an enriched protein on Golgi membrane vesicles that were immunoisolated using an epitope-tagged version of Sed5p (Inadome *et al*., 2005). They referred to this protein as Svp26, for Sed5p compartment vesicle protein of 26 kDa. In the current report, we refer to YHR181w as Erv26p or Svp26/Erv26p.

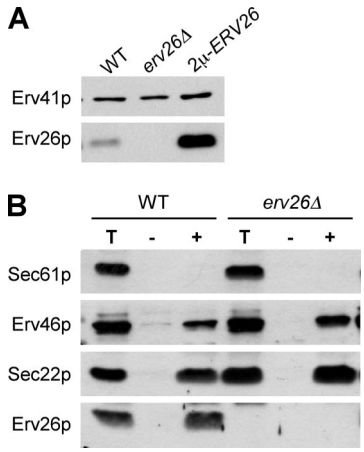

Figure 1. Erv26p is packaged into COPII-derived vesicles. (A) Cell extracts prepared from wild-type (CBY740), *erv26*Δ (CBY1480), and 2μ *ERV26* (CBY1913) strains were immunoblotted with anti-Erv26p serum to test antibody specificity. Erv41p serves as a loading control. (B) In vitro COPII budding reactions with membranes prepared from wild-type (CBY740) and *erv26*Δ (CBY1480) strains. Lanes contain one-tenth of a total reaction (T) and budded vesicles isolated after incubations in the absence (−) or presence (+) of COPII proteins. Samples were immunoblotted with antiserums specific for Sec61p (ER resident), Erv46p (ER vesicle protein), Sec22p (ER/Golgi SNARE protein), and Erv26p.

To investigate the cellular location and function of Erv26p, polyclonal antibodies were prepared against a GST-Erv26p fusion protein. As seen in Figure 1A, this antiserum recognized a 26-kDa protein by immunoblot that was not detected in an *erv26*Δ strain and was overproduced in a strain containing a 2μ *ERV26* plasmid. We next assessed the packaging efficiency of Erv26p into COPII vesicles. Formation of ER-derived transport vesicles can be reconstituted in vitro by incubation of ER membranes with purified COPII components. The in vitro COPII-dependent budding assay also recapitulates the selective incorporation of cargo that occurs during protein export from the ER (Barlowe *et al*., 1994). After separation of COPII vesicles from ER donor membranes, selective packaging can be monitored by immunoblot analysis of specific proteins in each fraction (Figure 1B). The ER-resident protein Sec61p was not packaged into the vesicle fraction, whereas the vesicle soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein Sec22p and the ER vesicle protein Erv46p were packaged efficiently. Both Sec22p and Erv46p are known to cycle between the ER and Golgi compartments (Otte *et al*., 2001). Notably, Erv26p was also efficiently packaged into COPII vesicles, and the extent of packaging was similar to other known vesicle proteins. In addition, this experiment demonstrated that the *erv26*Δ mutation does not reduce the level of COPII budding in vitro when packaging of Erv46p and Sec22p were monitored. Furthermore, the level of [^{35}S]glycopro-α-factor (gpαf) budding and transport to the Golgi complex in vitro was not significantly different in *erv26*Δ membranes compared with membranes prepared from a wild-type strain (our unpublished data). Together, these results indicate that Erv26p is efficiently packaged into

COPII vesicles but is not generally required for vesicle formation or transport to the Golgi complex.

Erv26p Is an Integral Membrane Protein That Cycles between the ER and Golgi Compartments

Sequence analysis of Erv26p predicts four transmembrane domains, with both N and C termini exposed to the cytosol (von Heijne, 1992). To determine the nature of Erv26p association with membranes, wild-type semi-intact cells were treated under conditions that extract peripherally bound membrane proteins (0.5 M NaCl), solubilize integral membrane proteins (0.1% Triton X-100), or release lumenal proteins (0.1 M Na_2CO_3, pH 11). After centrifugation of treated semi-intact cells, the supernatant and pellet fractions were inspected by immunoblot (Figure 2A). The fractionation profile of Erv26p was identical to the integral membrane protein Erv41p (Otte *et al*., 2001) and not the peripheral membrane protein Sec23p (Hicke and Schekman, 1989). Based on this fractionation behavior, we conclude that Erv26p is an integral membrane protein.

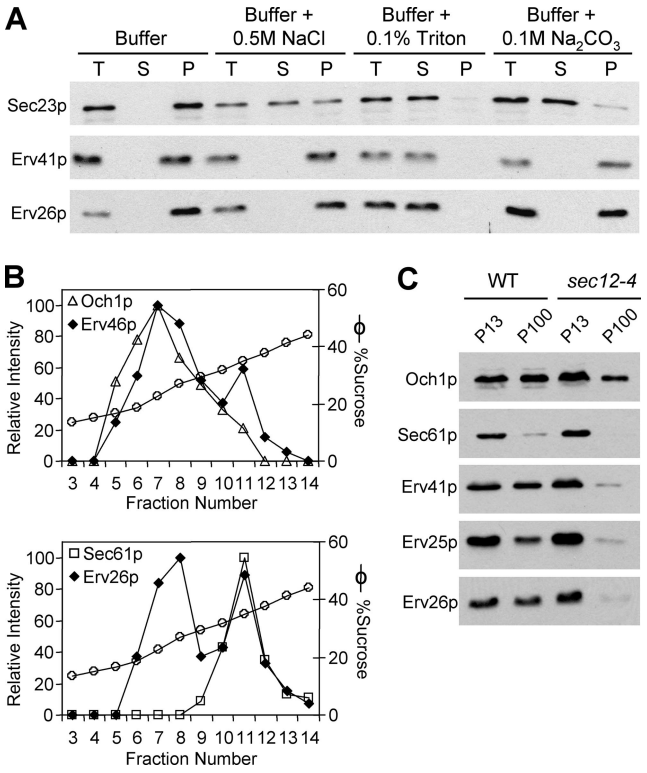

Figure 2. Erv26p is an integral membrane protein that cycles between ER and Golgi membranes. (A) Wild-type (CBY740) semi-intact cells were treated with buffer alone, buffer containing high salt (0.5 M NaCl), detergent (0.1% Triton X-100), or carbonate (0.1 M Na_2CO_3, pH 11), and centrifuged at 100,000 × *g*. The resulting supernatant fluid (S) and pellet factions (P) were immunoblotted for Erv41p (integral membrane protein), Sec23p (peripheral membrane protein), and Erv26p. (B) Sucrose gradient fractionation of a wild-type (CBY740) lysate on an 18–60% density gradient. After centrifugation, fractions were collected from the top of the gradient and analyzed by immunoblot. Relative levels of Och1p (Golgi marker), Sec61p (ER marker), Erv46p (ER vesicle protein), and Erv26p in each fraction were quantified by densitometry of immunoblots. (C) ER (p13) and Golgi (p100) membrane fractions prepared from wild-type (RSY248) and *sec12-4* (RSY263) strains after shift to 37°C for 45 min. Note that Erv26p shifts to the ER fraction in the *sec12-4* strain.

Integral membrane COPII vesicle proteins could be components of the ER/Golgi transport machinery or secretory proteins en route to their final cellular location. To determine the subcellular localization of Erv26p, we examined the distribution of Erv26p after resolution of membrane organelles on sucrose gradients (Figure 2B). Under steady state conditions, ~60% of the Erv26p cofractionated with the Golgi marker protein Och1p and ~40% with the ER marker Sec61p. This subcellular distribution pattern was similar to other COPII vesicles proteins including Emp24p, Erv46p and ER/Golgi SNARE proteins (Schimmoller *et al*., 1995; Otte *et al*., 2001; Cao and Barlowe, 2000).

To test whether Erv26p dynamically cycles between the ER and Golgi compartments in vivo, we monitored the fate of this protein in a *sec12* mutant strain. The *sec12* mutation blocks COPII budding when shifted to a restrictive temperature (Kaiser and Schekman, 1990); therefore, if Erv26p actively cycles between the ER and Golgi, we would expect it to accumulate in the ER under a *sec12* block. ER membranes can be effectively separated from Golgi membranes by differential centrifugation of gently lysed yeast cells (Wooding and Pelham, 1998). As shown in Figure 2C, Erv26p was found in both the ER and Golgi fractions under normal growth conditions but was shifted to the ER fraction after a *sec12* block. Other itinerant ER–Golgi proteins (Erv41p and Erv25p) displayed a similar redistribution to the ER, whereas Och1p (Golgi marker) and Sec61p (ER marker) were only mildly altered. Based on these results, we conclude that Erv26p actively cycles between early compartments of the secretory pathway and is not a transiting secretory protein en route to a distal site.

Strains Lacking Erv26p Exhibit a Selective Transport Defect

Examination of *erv26*Δ growth rates and in vitro transport rates of gpαf did not indicate any general defects in intracellular trafficking. We next performed pulse-chase experiments to assess the kinetics of secretory protein biogenesis in vivo. Cells were pulsed for 7 min with [^{35}S]methionine and [^{35}S]cysteine to label newly synthesized proteins. Excess unlabeled amino acids were added for the chase phase, and the maturation rates of Gas1p, CPY, and ALP were monitored after immunoprecipitation with specific antibodies to each protein (Figure 3). In wild-type strains, Gas1p initially appears in the ER as a 105-kDa GPI-anchored protein, is progressively glycosylated during passage through the

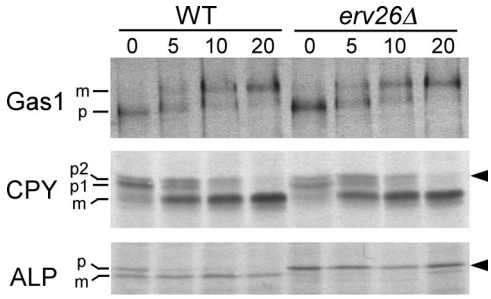

Figure 3. Cells lacking Erv26p accumulate pro-ALP. Wild-type (CBY740) and *erv26*Δ (CBY1480) strains were pulsed with ^{35}S-labeled amino acids and chased for indicated times. Cell extracts were prepared, and Gas1p, CPY, and ALP were immunoprecipitated from a common extract. Proteins were resolved on 10% polyacrylamide gels, and labeled species were visualized by fluorography. Arrowheads indicate the position of the 66-kDa molecular weight marker.

Golgi complex, and delivered to the plasma membrane as the 125-kDa mature form (Nuoffer *et al*., 1991). The biogenesis of vacuolar CPY begins in the ER as a 67-kDa p1 precursor, which is modified in the Golgi to produce the 69-kDa p2 form. On reaching the vacuole, p2 CPY is proteolytically processed to produce the mature 61-kDa form (Stevens *et al*., 1984). In the maturation of vacuolar ALP (encoded by the *PHO8* gene), the 66-kDa ER pro-form of ALP is transported through the Golgi and proteolytically processed in the vacuole to generate the 60-kDa mature form (Klionsky and Emr, 1989). In comparing the maturation kinetics of these three secretory proteins, we observed a prominent delay in the maturation of ALP in an *erv26*Δ strain compared with the wild type. In the mutant, the synthesis and stability of pro-ALP was not affected, but only ~7% of the initial pro-ALP had been processed to the mature form after 20 min. After extending the chase period out to 60 min, ~38% of the labeled ALP was detected in the mature form (Supplemental Figure 1), indicating a severe delay but not a complete block in ALP maturation. The ALP defect was cargo specific given that Gas1p and CPY were transported at normal rates in the *erv26*Δ strain. The observed delay was probably not due to a defect in processing of pro-ALP to mature ALP because both pro-CPY and pro-ALP are processed in a Pep4p-dependent manner (Hemmings *et al*., 1981). CPY processing was clearly not delayed in the *erv26*Δ mutant. Therefore, loss of Erv26p seems to cause a selective transport defect in which pro-ALP is not delivered efficiently to the vacuole.

The Pro-Form of ALP Accumulates in the ER of erv26Δ Cells

To investigate the intracellular location of pro-ALP in *erv26*Δ strains, we first measured steady-state levels of pro-ALP and mature ALP in whole cells by immunoblot analysis (Figure 4A). In wild-type strains, most cellular ALP was detected as the mature vacuolar form, as expected. However, in *erv26*Δ strains, approximately one-half of the cellular ALP was present as the precursor form. The pro-ALP that accumulated in *erv26*Δ cells seemed to be stable and associated with the membrane fraction. This result is consistent with the delay in ALP maturation observed in our pulse-chase analysis.

Membranes prepared from wild-type and *erv26*Δ strains were resolved on sucrose density gradients to determine the subcellular location of the accumulating pro-ALP. As shown in Figure 4B, the ER (Sec61p) and Golgi (Och1p) organelle markers were cleanly separated by this fractionation procedure. However, vacuolar proteins (Vam3p and mature ALP) were detected in two peaks; one peak near the top of the gradient and a second peak coincident with ER membranes. The vacuolar membranes that migrate deep into the sucrose gradient probably represent fragmented vacuoles from this lysis procedure. In the *erv26*Δ cells (Figure 4C), organelle marker proteins (Sec61p, Och1p, and Vam3p) migrated as observed in a wild-type strain. However, the pro-ALP that accumulated in *erv26*Δ was entirely coincident with the ER marker protein (Sec61p) and does not migrate with Golgi membranes or the vacuolar membranes in top fractions of the sucrose gradient. Although this gradient procedure was not ideal for resolving vacuolar membranes, these results indicate that the pro-ALP form in *erv26*Δ cells accumulates in the ER.

As an independent method to evaluate pro-ALP localization in cells lacking Erv26p, we performed immunofluorescence experiments to visualize pro-ALP in wild-type and *erv26*Δ strains. For these experiments, an HA-tagged version of ALP was constructed by appending the triple HA epitope

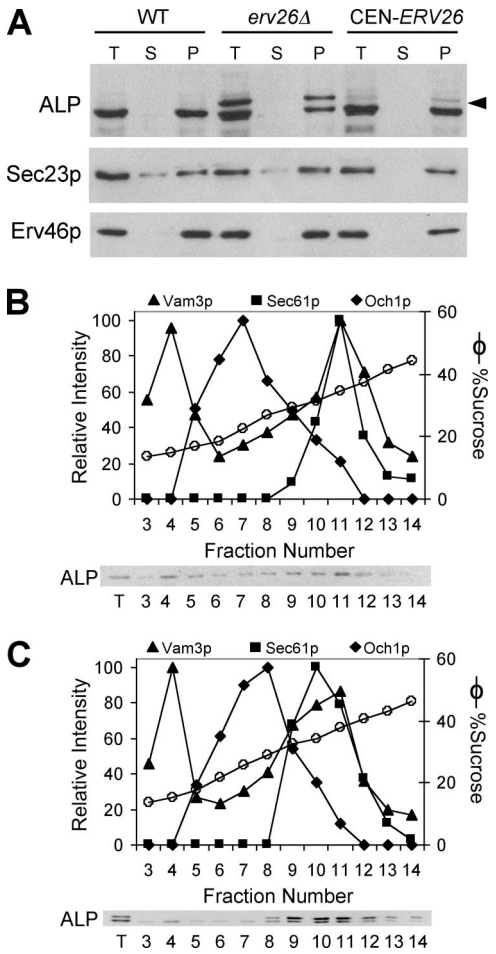

Figure 4. Pro-ALP accumulates in the ER of *erv26*Δ cells. (A) Immunoblot analysis of total lysates (T), supernatant fractions (S), and pellet fractions (P) from wild-type (CBY740), *erv26*Δ (CBY1480), or *erv26*Δ/*CEN-ERV26* (CBY1912) strains. Proteins were resolved on an 8% polyacrylamide gel, and the arrowhead indicates the position of the 66-kDa molecular weight marker. (B) Sucrose gradient fractionation of wild-type or (C) *erv26*Δ cell lysates followed by immunoblot analysis of individual fractions. Note in wild type that mature ALP cofractionates with the vacuolar marker Vam3p, whereas in the *erv26*Δ strain pro-ALP comigrates with the ER marker Sec61p.

to the extreme C terminus of ALP (Longtine *et al*., 1998). In this ALP-HA fusion, conversion of pro-ALP to mature ALP removes the HA-tag and allows us to specifically detect the pro-form when probed with anti-HA mAb (Figure 5A). Immunoblot of wild-type and *erv26*Δ whole cells demonstrated that pro-ALP-HA accumulates in the absence of Erv26p. Immunofluorescence images generated from double staining wild-type and *erv26*Δ strains with anti-Kar2p (ER marker) and anti-HA revealed that pro-ALP-HA colocalized with Kar2p in the *erv26*Δ mutant (Figure 5B). In wild-type strains, a lower signal of ALP-HA was detected, and the staining seemed more punctate and less perinuclear. These HA-staining puncta may represent pro-ALP-HA exiting the ER at transitional sites (Rossanese *et al*., 1999) or prevacuolar forms localized to Golgi membranes (Wooding and Pelham, 1998). Importantly, the immunofluorescence results confirm that pro-ALP-HA builds up in the ER of *erv26*Δ cells.

We next assessed whether this ER accumulation of pro-ALP in *erv26*Δ cells resulted in a compensatory activation of

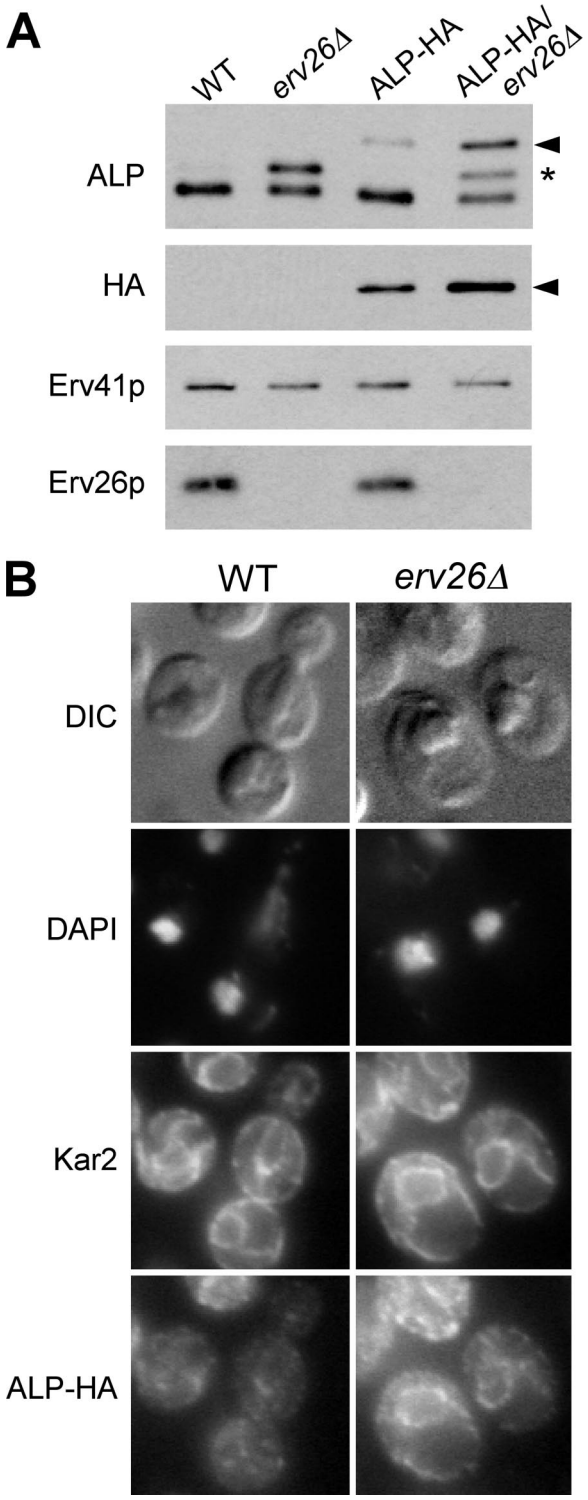

Figure 5. Immunofluorescence microscopy of ALP-HA shows ER localization in *erv26*Δ cells. (A) Immunoblot analysis of ALP and endogenously tagged ALP-HA in wild-type (CBY740), *erv26*Δ (CBY1480), ALP-HA in wild-type (CBY2076), and ALP-HA in *erv26*Δ (CBY2077) strains. Blots were probed with anti-ALP polyclonal antibodies or anti-HA monoclonal antibodies. The pro-ALP-HA that accumulates in the *erv26*Δ strain (indicated by arrowhead) is partially degraded to untagged pro-ALP (indicated by asterisk). (B) Double label immunofluorescence of wild-type (CBY2076) and *erv26*Δ (CBY2077) cells by using anti-Kar2p (ER localized) and anti-HA (pro-ALP-HA). Differential interference contrast (DIC) indicates cell boundaries and 4,6-diamidino-2-phenylindole (DAPI) stains nuclei. Note significant overlap in staining pattern of the ER-protein Kar2p and pro-ALP-HA in *erv26*Δ cells.

Table 2. UPR induction

Strain	-Fold UPR
Wild type	1
*erv26*Δ	1.3
*erv41*Δ	>1
*erv29*Δ	3.5
*erv25*Δ	5.8
*erv14*Δ	2.8

the UPR pathway. The UPR controls ER homeostasis through transcriptional activation of several genes involved in ER folding, degradation, and export (Travers *et al*., 2000). To measure the level of transcriptional activation from the unfolded protein response element (UPRE), we integrated the UPRE-GFP reporter construct into various yeast strains (Table 2). Compared with the wild type, *erv26*Δ cells displayed a mild 1.3-fold activation of the UPR. Other *erv* mutations that cause an ER accumulation of selected secretory proteins, including *erv14*Δ, *erv25*Δ, and *erv29*Δ, produced varying levels of UPR. These results indicate that the UPR is activated in cells that fail to efficiently export secretory proteins from the ER. The moderate UPR exhibited by *erv26*Δ mutants is consistent with the observed accumulation of pro-ALP in the ER.

Erv26p Directs Pro-ALP into COPII Vesicles

Based on our pulse-chase experiments, subcellular localization studies and UPR readout in *erv26*Δ mutants, we hypothesized that Erv26p directs pro-ALP into COPII vesicles during transport from the ER. To test this idea, three lines of investigation were pursued. First, we assessed the gene dosage relationship between ALP (*PHO8*) and *ERV26* on biogenesis of mature ALP. Second, we inhibited Erv26p activity in COPII budding assays to determine whether Erv26p is directly required for export of pro-ALP from the ER. Third, we immunoisolated pro-ALP from detergent-solubilized ER membranes to monitor potential associations with Erv26p.

If Ev26p performs a receptor-like role in ER export of pro-ALP, we reasoned that overexpression of ALP should saturate Erv26p-dependent export from the ER. In Figure 6, we observed that cells harboring a 2μ plasmid overexpressing ALP from the *PHO8* gene produced an ~14-fold increase in cellular ALP. Notably, this overexpression caused a significant increase in cellular levels of pro-ALP and ~65% of overexpressed ALP remained in the unprocessed form. Transformation of this ALP overexpression strain with a 2μ plasmid containing *ERV26* increased cellular levels of Erv26p ~10-fold compared with wild-type strains. Importantly, the overexpression of Erv26p promoted ALP processing such that only 35% of total ALP persisted in the pro-form, and there was a corresponding increase in the cellular level of mature ALP. We speculate that other cellular factors become limiting for ALP biogenesis even when Erv26p expression was elevated. Regardless, this reciprocal connection between ALP processing and Erv26p expression level in an otherwise wild-type background supports a model wherein Erv26p performs a direct role in ALP export from the ER.

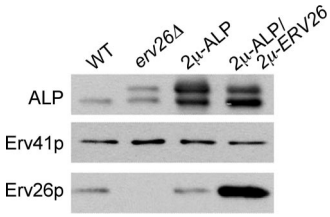

Figure 6. Expression level of Erv26p influences ALP processing. Immunoblot analysis of wild-type (CBY740), *erv26*Δ (CBY1480), 2μ *PHO8* (CBY2140), and 2μ *PHO8*/2 μ *ERV26* (CBY2194) strains. Blots were probed for ALP, Erv41p (loading control), and Erv26p. Note overexpression of *ERV26* in CBY2194 increases the level of mature ALP.

To determine whether Erv26p was directly involved in the packaging of pro-ALP into COPII vesicles, we performed in vitro vesicle budding assays with wild-type membranes under conditions that inhibit Erv26p function. Our initial observations indicated that addition of the anti-Erv26p antibodies to in vitro budding reactions specifically blocked packaging of Erv26p into COPII vesicles but did not affect the packaging of other vesicle proteins (Figure 7A). Presumably antibody binding to the C-terminal residues of Erv26p sterically hinders coat subunit binding to Erv26p and prevents its packaging into vesicles as has been observed for other COPII vesicle proteins (Rowe *et al*., 1998; Belden and Barlowe, 2001). When budding reactions were preformed using ER membranes that contained the HA-tagged version of pro-ALP (Figure 7B), COPII-dependent packaging of pro-ALP-HA was observed. However, addition of anti-Erv26p antibodies depleted both Erv26p and pro-ALP-HA from COPII-formed vesicles. Importantly, the packaging efficiency of other vesicles proteins was not decreased when Erv26p function was neutralized. These results indicate that

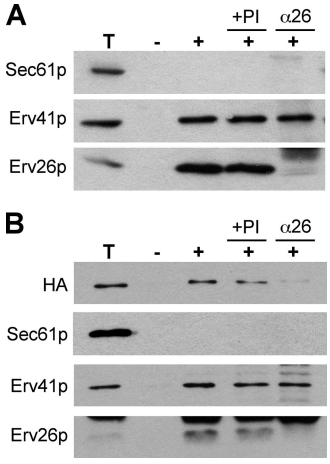

Figure 7. Erv26p is required for efficient packaging of pro-ALP into COPII vesicles. Reconstituted COPII budding reactions from wild-type ER membranes (CBY740) incubated with (+) or without (−) purified COPII proteins and in the presence of preimmune (+PI) or anti-Erv26p (α26) antibodies. Lanes labeled T represent one-tenth of the total reactions. (B) COPII budding as in A by using wild-type ER membranes expressing ALP-HA (CBY2076) in the presence of preimmune (+PI) or anti-Erv26p (α26) antibodies. Where indicated, antibodies were added to a final concentration of 0.1 mg/ml. Erv41p serves as a positive control for COPII vesicle formation. Note specific inhibition of Erv26p and pro-ALP-HA packaging by anti-Erv26p antibodies.

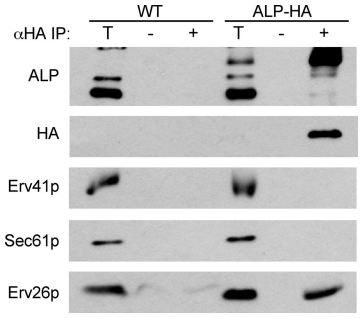

Figure 8. ALP associates specifically with Erv26p. Microsomes from untagged (CBY740) and HA-tagged (CBY2076) strains were solubilized with digitonin and tagged proteins immunoprecipitated with anti-HA monoclonal antibodies. Gel lanes compare 10% of total membrane extracts (T) with immunoprecipitates (IP) in the presence (+) or absence (−) of anti-HA antibodies. Immunoblots were probed with antibodies against ALP, HA, Erv41p, and Sec61p (integral membrane controls) and Erv26p. Note specific coprecipitation of Erv26p with ALP-HA only in the tagged strain.

Erv26p function is specifically and directly required for efficient incorporation of pro-ALP into COPII vesicles.

To test whether Erv26p and pro-ALP are physically associated in ER membranes, a native coimmunoprecipitation approach was undertaken with the HA-tagged version of pro-ALP. Microsomes from HA-tagged and untagged strains were solubilized in digitonin, and pro-ALP-HA was immunoprecipitated with monoclonal HA antibody. As shown in Figure 8, Erv26p coprecipitated with HA-ALP in the tagged strain, whereas no Erv26p was precipitated from the untagged wild-type microsomes. As controls, neither Erv41p nor Sec61p were precipitated under these conditions, indicating that the association of Erv26p with ALP-HA was specific. Approximately 56% of the total pro-ALP-HA was recovered in this anti-HA immunoprecipitation, and ~7% of the total Erv26p was detected in complex with pro-ALP-HA in this experiment. In complementary experiments, we found that ALP-HA could be coprecipitated with Erv26p when anti-Erv26p–specific antibodies were used (our unpublished data). Collectively, these results indicate that Erv26p and pro-ALP are assembled into an oligomeric complex in ER membranes that can be recovered from detergent-solubilized membranes.

DISCUSSION

Several lines of evidence indicate that the COPII coat complex selects cargo proteins for export from the ER (Lee *et al*., 2004). However, the mechanisms by which this coat complex can accommodate such diversity in secretory cargo are not known. In this report, we identify and characterize Erv26p, a novel and conserved component of the ER sorting machinery that is required for efficient export of specific secretory cargo. We initially detected Erv26p on ER-derived transport vesicles and observed that this protein is distributed between the ER and Golgi compartments. In *erv26*Δ mutant cells, the secretory pathway continued to operate but the pro-form of vacuolar ALP accumulated in the ER. In vitro experiments demonstrated that export of pro-ALP from the ER depended on Erv26p and that an Erv26p/pro-ALP complex could be isolated from solubilized microsomal membranes. Based on these findings, we propose that Erv26p couples pro-ALP and probably other cargo proteins to the COPII coat for efficient export from the ER. Once Erv26p-

cargo complexes reach early Golgi compartments, bound cargo is presumably released and the empty receptor recycles back to the ER for subsequent rounds of export.

Erv26p is encoded by the open reading frame YHR181w on chromosome VIII. Database alignments indicate that Erv26p is a widely conserved protein with other eukaryotic species having a single predicted homologue ranging from 54% (*Candida albicans*) to 35% (*D. melanogaster*) and 39% (*Homo sapiens*) amino acid identity. No known functions have been reported for these homologous proteins. In yeast, a recent publication identified the gene product of YHR181w as one of 29 enriched proteins on immunoisolated Sed5p-containing membranes and called this protein Svp26, for Sed5p compartment vesicle protein of 26 kDa (Inadome *et al*., 2005). This study indicated that Svp26 localizes to early Golgi compartments and associates with the Golgi localized mannosyltransferase protein Ktr3p. Interestingly, Ktr3p is a type II membrane protein that was partially mislocalized to the ER in *svp26*Δ mutants. Based on their observations, these authors propose that Svp26 is a Golgi localized protein that functions to retain Ktr3p in the Golgi complex. Many of our findings are in accord with this study, although our results indicate that Svp26/Erv26p cycles between the ER and Golgi compartments, whereas Inadome and colleagues report that this protein is confined to early Golgi membranes. This discrepancy could be explained by a difference in the behavior of tagged Svp26-HA described in their report compared with the endogenous Erv26p protein monitored in our study. If we assume this protein cycles, an alternative interpretation of the results could be that Ktr3p depends on Svp26/Erv26p for export from the ER and that deletion causes an ER accumulation of Ktr3p as was observed (Inadome *et al*., 2005). However, further experimentation will be required to determine whether Svp26/Erv26p functions in ER export or Golgi retention of Ktr3p.

Why would an integral membrane secretory protein, such as ALP, depend on an adaptor protein for ER export? Yeast ALP has the topology of a type II integral membrane protein and consists of a conserved lumenal catalytic domain, a single transmembrane-spanning segment, followed by an NH_2-terminal cytoplasmic tail sequence of ~30 amino acids (Klionsky and Emr, 1989). With an exposed cytoplasmic tail sequence, ALP could in principle bind directly to the COPII coat complex. For example, other transmembrane secretory proteins, such as vesicular stomatitis virus-G (Nishimura and Balch, 1997) and Gap1p (Malkus *et al*., 2002), contain cytoplasmic sorting signals that are required for efficient ER export and interact with COPII subunits. However, it may be important to note that even though this phosphatase catalytic domain is widely conserved in nature (Coleman, 1992), ALP occurs as a soluble secreted protein in some species (Inouye and Beckwith, 1977) and a GPI-anchored cell surface protein in others (Howard *et al*., 1987). Therefore, efficient ER export of ALP in eukaryotic cells may have initially evolved for a soluble form of the enzyme without cytoplasmically exposed residues. In this case, a transmembrane adaptor may be required for linkage to cytoplasmic coat proteins. It is noteworthy that the vacuolar localization of yeast ALP is distinct from the cell surface forms of GPI anchored ALP in mammals and in other species. In addition, the cytoplasmic tail sequence of yeast ALP contains a defined sorting signal for AP3 dependent transport from the Golgi complex to the vacuole (Piper *et al*., 1997; Cowles *et al*., 1997; Vowels and Payne, 1998) that is not present in the mammalian enzymes. Therefore, after Erv26p-dependent export from the ER, we speculate that yeast has evolved a mechanism to divert ALP to the vacuole instead of the cell surface, presumably for a selective advantage. It will be interesting to test whether the GPI-anchored forms of mammalian ALP depend on the Erv26p homologue for efficient ER export.

In yeast, export of pro-ALP from the ER was not absolutely dependent on Erv26p. Although we observed an ~10-fold reduction in the rate of ALP transport to the vacuole, about one-half of intracellular ALP was localized to the vacuole in *erv26*Δ strains during a logarithmic stage of growth. We also note that the pro-ALP accumulating in the ER of *erv26*Δ strains was stable (Figure 3) and that *erv26*Δ strains exhibited only a mild UPR (Table 2). Based on these observations, we propose that Erv26p is not necessary for the folding or assembly of ALP and that bulk flow movement of fully folded ALP out of the ER can explain the reduced transport rate in *erv26*Δ mutants (Malkus *et al*., 2002). Once ALP reaches Golgi compartments, AP3-dependent delivery to the vacuole (Piper *et al*., 1997; Cowles *et al*., 1997; Vowels and Payne, 1998) and Pep4p-dependent maturation (Klionsky and Emr, 1989) seem to proceed at wild-type rates. Similar reductions in the transport rate of soluble secretory cargo have been observed in the absence of other specific ER cargo receptors, including Erv29p-dependent export of yeast gpαf and CPY (Belden and Barlowe, 2001) as well as the ERGIC53-dependent export of coagulation factors V/VII and procathepsin Z in animal cells (Nichols *et al*., 1998; Appenzeller *et al*., 1999). Some type I integral membrane cargo are also known to depend on cycling cargo receptors such as Erv14p (Powers and Barlowe, 1998), Emp47p (Sato and Nakano, 2003), and Vma21p (Malkus *et al*., 2004) for ER export. Currently, it is not known whether the cargo adaptor mechanism will be a common feature in ER export, because other transmembrane cargo proteins can bind directly to COPII subunits and are exported without apparent adaptors (Lee *et al*., 2004). Even some soluble secretory proteins, such as amylase and chymotrypsinogen in exocrine cells, do not seem to rely on cargo adaptors and depart the ER in a bulk flow manner (Martinez-Menarguez *et al*., 1999). Nonetheless, given the diversity in secretory proteins and the limited number of cargo proteins that have been thoroughly examined at the ER export stage, we speculate that ER cargo adaptors will be a relatively common feature.

Erv26p was detected in a complex with pro-ALP from solubilized membranes and COPII-dependent packaging of pro-ALP was inhibited in vitro and in vivo when Erv26p function was compromised. We propose a basic model to explain these observations in which Erv26p contacts both pro-ALP and COPII subunit(s) to direct cargo into ER vesicles. Moreover, we hypothesize that the formation of this pro-ALP/Erv26p/COPII complex is regulated in a manner to promote assembly at ER exit sites and then disassembled in post-ER compartments. It should be interesting to explore this model by determining the sorting signal(s) present in pro-ALP necessary for Erv26p-dependent ER export. Current experimentation suggests that the ER export information does not reside in cytoplasmic tail sequences of pro-ALP, although this region is needed for efficient targeting to the vacuole (Klionsky and Emr, 1990; Piper *et al*., 1997; our unpublished observations). A combined genetic and biochemical analysis of Erv26p in yeast should provide insight on the amino acid residues necessary for interaction with pro-ALP and COPII. Given the overall conservation of Erv26p in nature, elucidation of this Erv26p-dependent export mechanism should contribute to our understanding on the biogenesis of very diverse classes of secretory proteins.

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