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

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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-solubilized 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. Cytoplasmic 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; Mossevossova 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.
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 erv26Δ strain were transported from the ER at wild-type rates. Interestingly, the secretory protein that accumulates in an erv26Δ 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) and bacteria (Ausubel et al., 1987) molecular genetic methods were used.

Plasmid Constructions

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) and bacteria (Ausubel et al., 1987) molecular genetic methods were used.

Strain Construction

The erv26Δ 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 (CGGGATCCTC TACTGGGCCT GACTGG) and SV26-E (CGGAATTCCT AAACAGCCAA CTTATCAAA) 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 antisera in rabbits (Covance, Denver, PA). The anti-Erv26p serum was typically used at a 1:10000 dilution for immunoblotting. Antibodies directed against Sec23p (Hicke and Schekman, 1989), Erv25p (Belden and Barlowe, 1996), Sec22p (String et al., 1992), Erv46p and Sec22p (Liu and Barlowe, 2002), Gaslp (Fankhauser and Conzemlin, 1991), CPY (Rothblatt et al., 1989), ALP (Haas et al., 1995), Vam3p (Wang et al., 2003), and Ochlp, 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).

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 (CCGGATCCCT AGCTGGTGAT TATGTG) and SV26-E (CCGAATTCCT AAACAGCCAA CTTATCAAA) 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 antisera in rabbits (Covance, Denver, PA). The anti-Erv26p serum was typically used at a 1:10000 dilution for immunoblotting. Antibodies directed against Sec23p (Hicke and Schekman, 1989), Erv25p (Belden and Barlowe, 1996), Sec22p (String et al., 1992), Erv46p and Sec22p (Liu and Barlowe, 2002), Gaslp (Fankhauser and Conzemlin, 1991), CPY (Rothblatt et al., 1989), ALP (Haas et al., 1995), Vam3p (Wang et al., 2003), and Ochlp, 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).

Table 1. Yeast strains used in this study

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<th>Strain</th>
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<td>BY4742 Research Genetics (Huntsville, AL)</td>
</tr>
<tr>
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<td>MATa his3 leu2 lys2 ura3 erv26ΔKAN6</td>
<td>16420 Research Genetics This study</td>
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<td>CBY1480 with pRS316-ERV26</td>
<td>This study</td>
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<td>CBY1480 with pRS426-ERV26</td>
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<td>MATa ura3-52 sec12-4</td>
<td>Kaiser and Schekman (1990)</td>
</tr>
</tbody>
</table>
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(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), Sec1p (ER marker), Van3p (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 erv26Δ (CBY1480) cells were grown in media with reduced sulfate to an OD600 of 0.4. Cells were harvested, washed, and resuspended at an OD600 of 3.0 in minimal media without sulfate. After preincubation at 30°C for 5 min, cultures were pulsed with [35S] GPiomix (GE Healthcare) at 25 μCi/OD600 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 Axiosplan 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 GFP signal was used to assess activation of the UPR.

RESULTS

Erv26p Is a Component of COPII-coated Vesicles

In previous studies, we identified several of the abundant membrane 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 -KXXX or -KQXXX. 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 immunosolated using an epitope-tagged version of Sed5p (Inadome et al., 2005). They referred to this protein as Svp26, for Sed5p compartment SNARE protein, and Erv46p (ER vesicle protein), Sec22p (ER/Golgi SNARE protein), and Erv26p.

Figure 1. Erv26p is packaged into COPII-derived vesicles. (A) Cell extracts prepared from wild-type (CBY740), erv26Δ (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 erv26Δ (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 antisera specific for Sec61p (ER resident), Erv46p (ER vesicle protein), Sec22p (ER/Golgi SNARE protein), and Erv26p.
COP II 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 luminal proteins (0.1 M Na2CO3, 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 Na2CO3, pH 11), and centrifuged at 100,000 × g. The resulting supernatant fluid (S) and pellet fractions (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.](image)

Integral membrane COP II 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 COP II 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 COP II 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 erv26Δ growth rates and in vitro transport rates of gpa1Δ 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 [35S]methionine and [35S]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 (CBY1740) and erv26Δ (CBY1480) strains were pulsed with [35S]-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.](image)
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 erv26Δ 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 erv26Δ 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 erv26Δ 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 erv26Δ 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 erv26Δ strains, approximately one-half of the cellular ALP was present as the precursor form. The pro-ALP that accumulated in erv26Δ 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 erv26Δ 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 erv26Δ 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 erv26Δ 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 erv26Δ 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 erv26Δ strains. For these experiments, an HA-tagged version of ALP was constructed by appending the triple HA epitope 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 erv26Δ whole cells demonstrated that pro-ALP-HA accumulates in the absence of Erv26p. Immunofluorescence images generated from double staining wild-type and erv26Δ strains with anti-Kar2p (ER marker) and anti-HA revealed that pro-ALP-HA colocalized with Kar2p in the erv26Δ 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 (Woodying and Pelham, 1998). Importantly, the immunofluorescence results confirm that pro-ALP-HA builds up in the ER of erv26Δ cells.

We next assessed whether this ER accumulation of pro-ALP in erv26Δ cells resulted in a compensatory activation of
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/H9004 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/H9004, erv25/H9004, and erv29/H9004, 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/H9004 mutants is consistent with the observed accumulation of pro-ALP in the ER.

**Table 2. UPR induction**

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**Erv26p Directs Pro-ALP into COPII Vesicles**

Based on our pulse-chase experiments, subcellular localization studies and UPR readout in erv26Δ 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 Erv26p 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 5.** Immunofluorescence microscopy of ALP-HA shows ER localization in erv26Δ cells. (A) Immunoblot analysis of ALP and endogenously tagged ALP-HA in wild-type (CBY740), erv26Δ (CBY1480), ALP-HA in wild-type (CBY2076), and ALP-HA in erv26Δ (CBY2077) strains. Blots were probed with anti-ALP polyclonal antibodies or anti-HA monoclonal antibodies. The pro-ALP-HA that accumulates in the erv26Δ strain (indicated by arrowhead) is partially degraded to untagged pro-ALP (indicated by asterisk). (B) Double label immunofluorescence of wild-type (CBY2076) and erv26Δ (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 erv26Δ cells.
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-HA (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 vesicle proteins was not decreased when Erv26p function was neutralized. These results indicate that 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 erv26Δ 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 immunosolated 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 svp26Δ 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 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 luminal catalytic domain, a single transmembrane-spanning segment, followed by an NH2-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 is 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 erv26Δ strains during a logarithmic stage of growth. We also note that the pro-ALP accumulating in the ER of erv26Δ strains was stable (Figure 3) and that erv26Δ 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 erv26Δ 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 gap1p and CPY (Belden and Barlowe, 2001) as well as the ERGIC53-dependent export of coagulation factors V/VII and procathespin Z in animal cells (Nichols et al., 1997; 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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