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Erp1p and Erp2p, Partners for Emp24p and Erv25p in a Yeast p24 Complex

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Six new members of the yeast p24 family have been identified and characterized. These six genes, named *ERP1–ERP6* (for Emp24p- and Erv25p-related proteins) are not essential, but deletion of *ERP1* or *ERP2* causes defects in the transport of Gas1p, in the retention of BiP, and deletion of *ERP1* results in the suppression of a temperature-sensitive mutation in *SEC13* encoding a COPII vesicle coat protein. These phenotypes are similar to those caused by deletion of *EMP24* or *ERV25*, two previously identified genes that encode related p24 proteins. Genetic and biochemical studies demonstrate that Erp1p and Erp2p function in a heteromeric complex with Emp24p and Erv25p.

INTRODUCTION

Transport between organelles of the secretory pathway is mediated by membrane-bound vesicles that bud from the surface of a donor membrane and specifically fuse with a target membrane (Palade, 1975). Genetic and biochemical studies have identified key proteins that mediate this process, many of which are conserved from yeast to mammalian cells (Rothman and Wieland, 1996; Schekman and Orci, 1996). In the early secretory pathway, between the endoplasmic reticulum (ER) and the Golgi apparatus, transport in the anterograde direction is mediated via vesicles coated with a protein complex termed COPII (Barlowe *et al.*, 1994; Kuehn and Schekman, 1997; Kaiser and Ferro-Novick, 1998). Anterograde transport is balanced by a retrograde transport pathway, mediated by COPI-coated vesicles, that facilitates the recovery of membrane, ER resident proteins and limiting vesicle targeting and fusion proteins back to the ER from later compartments of the secretory pathway (Letourneur *et al.*, 1994; Cosson and Letourneur, 1997). COPI-coated vesicle populations have also been implicated in intra-Golgi anterograde and retrograde transport (Orci *et al.*, 1997) and endocytosis (Daro *et al.*, 1997) and have been observed to bud directly from the ER (Bednarek *et al.*, 1995).

Recent biochemical and genetic approaches in yeast, mammalian systems, and frog have identified a conserved family of 23- to 27-kDa type I transmembrane proteins that have been termed the p24 family. The function of these proteins is currently unknown, but it is clear from several lines of evidence that p24 proteins play an important role in the early secretory pathway (Wada *et al.*, 1991; Holthuis *et al.*, 1995; Schimmöller *et al.*, 1995; Stamnes *et al.*, 1995; Belden and Barlowe, 1996; Blum *et al.*, 1996; Fiedler *et al.*, 1996; Sohn *et al.*, 1996; Rojo *et al.*, 1997; Dominguez *et al.*, 1998). Members of this protein family all contain a single membrane-spanning domain with a short cytoplasmic C-terminal tail that, in a subset of p24 proteins, contains a motif related to the KKXX or K(X)KXX motif previously implicated in retrieval to the ER (Nilsson *et al.*, 1989; Jackson *et al.*, 1990). Consistent with these observations, some p24 proteins have been demonstrated to bind subunits of both COPI and COPII vesicle coats in vitro (Fiedler *et al.*, 1996; Sohn *et al.*, 1996; Dominguez *et al.*, 1998). p24 proteins have been found in both COPI and COPII vesicles in addition to ER and Golgi

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membranes (Schimmöller *et al.*, 1995; Stamnes *et al.*, 1995; Belden and Barlowe, 1996; Sohn *et al.*, 1996; Rojo *et al.*, 1997; Dominguez *et al.*, 1998).

In the budding yeast *Saccharomyces cerevisiae* two members of the p24 family, encoded by the *EMP24* and *ERV25* genes, have been previously identified (Schimmöller *et al.*, 1995; Belden and Barlowe, 1996). Cell fractionation experiments have demonstrated that Emp24p is predominantly ER localized with some Emp24p in the Golgi apparatus (Schimmöller *et al.*, 1995). Analysis of the steady-state distribution of Erv25p by indirect immunofluorescence has revealed a similar localization. Emp24p and Erv25p interact with one another in stoichiometric amounts in a complex in ER-derived membranes (Belden and Barlowe, 1996). In addition, both Emp24p and Erv25p are major proteins in COPII-coated vesicles. Furthermore, Emp24p and Erv25p are dependent on each other for stability and incorporation into COPII-coated vesicles (Belden and Barlowe, 1996).

Neither *EMP24* nor *ERV25* is an essential gene, but strains containing deletions in either gene exhibit delays in the ER-to-Golgi transport of a subset of secretory cargo molecules (Schimmöller *et al.*, 1995; Belden and Barlowe, 1996). These observations, taken together with the type I topology of p24 proteins, and the binding of vesicle coat proteins to their cytoplasmic tails in vitro, have led to the suggestion that members of the p24 family may function as receptors or adaptors for cargo in the early secretory pathway (Schimmöller *et al.*, 1995; Stamnes *et al.*, 1995; Belden and Barlowe, 1996). In this model the short cytosolic C-terminal tails of p24 proteins would interact with vesicle coat proteins, whereas the larger lumenal domains, and perhaps transmembrane regions, might interact with cargo molecules. There is currently no direct evidence in support of this hypothesis, however. A further possibility is that p24 proteins play a role in vesicle biogenesis. However, using an in vitro assay for the formation of ER transport vesicles, Belden and Barlow (1996) have demonstrated that membranes derived from *emp24*, *erv25*, and even *emp24/erv25* double mutants exhibit normal vesicle formation characteristics, although genetic experiments implicate at least Emp24p in the fidelity of ER-derived vesicle formation (Stamnes *et al.*, 1995; Elrod-Erickson and Kaiser, 1996). A further possibility is that p24 proteins may play a structural role in the early secretory pathway.

In this study we have identified and characterized six additional p24 family members in *S. cerevisiae*. We have called these genes *ERP1–ERP6* (for Emp24p- and Erv25p-related proteins). We find that deletion of two of these genes, called *ERP1* and *ERP2*, results in phenotypes similar to those caused by deletion of *EMP24* or *ERV25*. Genetic and biochemical experiments suggest that Emp24p, Erv25p, Erp1p, and Erp2p function together in a heteromeric complex.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Media

Yeast strains used in this study are listed in Table 1. Standard yeast genetic and molecular techniques were used. Yeast cells were grown in either YPD or YNB media supplemented as necessary (Kaiser *et al.*, 1994). The p24 deletion mutants were generated by replacing each ORF with the *HIS3* gene in a haploid segregant of YPH274 (DHY9) using standard PCR techniques. Primer sequences were GAACAGATTGTTGGCGCTTTCTTCCTTATCGCCTCAATCTGAAAGGATCTAGATTTGCCACGTTTTAAGAGCTTGGT and ATTGAAACAACGAAATTCTCATGTATGCCTGCTAAGGATTCAATTTTTTGATATGTACGGTCGAGTTCAAGAGAAAA (*ERP6*, YGL002w), GTGTAATACTACTAGTTACTTAAGGGAACACATCAAGCATTCGGTGTCTCACAGGCTACTCGTTTTAAGAGCTTGGT and AGCTCATTGCACCGATTGCACCGATTCGTAATGAGCGATATATAAACTCTATATAACAAACATGGTATATCGAGTTCAAGAGAAAA (*ERP5*, YHR110w), TACTTGGTGAAACTGCAATCTTAATAGTATCCCTCCGCACAAAAATACACACGCATAAGGCGTTTTAAGAGCTTGGT and CGTGTATGTAATCGTATGTAGGCAAAAGTAAATAGATATGAACTACATTTTCCTGCTTTACTCGAGTTCAAGAGAAAA (*EMP24*), AGCTGGGGCCTACCTTTATAGTTAGTTGTATAACTCAGTTGATCTCATAAGTGAAAAGCAAAAAAAGGCGTTTTAAGAGCTTGGT and ATAGTCGAGTACATATGCAATTATAGGGTAAGCTGATACACAAATGCATGGTGTGGTCCTCTTCCTTTGCTCGAGTTCAAGAGAAAA (*ERV25*), TGAAGTAGTTTCACGAGCGCTAGTGAAGTTTTTTCATAAATATATATTCCCAATCATCGCTAGGAATTCGTTTTAAGAGCTTGGT and TCATGATACAAAAAAGTGCCTTCCCCTTAGCACAGCTGATCAACAATTTTAAGAGCTTGAAAAGCAACTCGAGTTCAAGAGAAAA (*ERP4*, YOR016C), AAGGCTCTTGATAGTTACCGTACTTGAAGGGACACTGTGAACTGACTAAAAAACTCCGTCCGTTTTAAGAGCTTGGT and TAAATATGAAGAACATATTCTCAAGTT-GATAGAAAATGCAGGAACAATACACAACTATTCGAGTTCAAGAGAAAA (*ERP3*, YDL018C), TCAGGTGACCCAAACTAGAATAAGATAAACAGTGGAGTACACTCATTGTAAAGGAAAACCCGTTTTAAGAGCTTGGT and AATATAAACCTAATTGTGTTCTATATTGCGGACATATATTTTTCGTAGATTGAAAAGTTCTCGAGTTCAAGAGAAAA (*ERP2*, YAL007), and CTCTATCTTCCCTCTGTAGTTTGGAAAAGTATTTAAAGGTAACCAATCTCTCTACTAATCCGTTTTAAGAGCTTGGT and CTTTAGCGGCTCTTATAAACTATAAATTTCTAGAAGATACATAAAAGGTTTTTAGTCTGATCGAGTTCAAGAGAAAA (*ERP1*, YAR002A). Underlined sequences are derived from the *HIS3* locus. The entire p24 ORF was precisely replaced in each case. The deletion of each gene was verified by PCR. Double, triple, and quadruple p24 mutants were created by PCR genotyping segregants from diploids generated by crossing haploid deletion strains. The *sec13-1* mutation in CKY45 was back-crossed three times into the YPH274 genetic background to generate strain ARY63 suitable for crossing with the p24 deletion mutants. The *sec13-1* locus in ARY63 was then genetically marked with *URA3* by cloning a region of *SEC13* downstream flanking sequence (+884 to +1277 from ATG) into vector YEP352 (Hill *et al.*, 1986) and integrating this construct at the *sec13-1* locus by linearizing with *Eag*I before transformation. Correct integration was verified by PCR analysis of genomic DNA. This *URA3* marker allowed us to follow the *sec13-1* locus in genetic crosses with the p24 deletion mutants independently of its temperature-sensitive phenotype. Overexpression of yeast p24 genes was achieved by cloning each p24 ORF behind the triose phosphate isomerase (TPI) promotor in a modified version of plasmid TPαH (Dean and Pelham, 1990) in which the *Eco*RI–*Sal*I cassette was replaced by the p24 ORFs amplified by PCR. Constructs were integrated into *ura3* after cleavage with *Xho*I.

Invertase Assay

Cells were grown to midlogarithmic phase in YPD medium, harvested, and washed, and 10 OD_{600} units of cells were transferred to

Table 1. Yeast strains used in this study

Strain	Genotype	Source
YPH274	*MAT***a**/*MATα ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ1/ trp-Δ1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1*	ATCC
CKY45	*MATα sec13-1 ura3-52 his4-619*	Chris Kaiser
ARY63	*MAT***a** *sec13-1 leu2-Δ1 ura3-52 his ade2-101 Derived from CKY45*	This study
W303-1a	*MAT***a** *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*	Malcolm Whiteway
DHY1	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERP6::HIS3*	This study
DHY2	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERP5::HIS3*	This study
DHY3	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 EMP24::HIS3*	This study
DHY4	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERV25::HIS3*	This study
DHY5	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERP4::HIS3*	This study
DHY6	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERP3::HIS3*	This study
DHY7	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERP2::HIS3*	This study
DHY8	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ ERP1::HIS3*	This study
DHY9	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 (Haploid segregant from YPH274)*	This study
ARY74	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERP3::HIS3 ERP4::HIS3*	This study
ARY76	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERP4::HIS3 ERP2::HIS3*	This study
ARY78	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERP3::HIS3 ERP2::HIS3*	This study
ARY79	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERP5::HIS3 ERP6::HIS3*	This study
ARY82	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERP5::HIS3 ERP1::HIS3*	This study
ARY84	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 EMP24::HIS3 ERV25::HIS3*	This study
ARY86	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 EMP24::HIS3 ERP1::HIS3*	This study
ARY88	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERV25::HIS3 ERP1::HIS3*	This study
ARY100	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERP6::HIS3 ERP1::HIS3*	This study
ARY101	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERP5::HIS3 ERP6::HIS3 ERP1::HIS3*	This study
ARY102	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 ERP2::HIS3 ERP3::HIS3 ERP4::HIS3*	This study
ARY103	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 EMP24::HIS3 ERV25::HIS3 ERP1::HIS3*	This study
ARY104	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1 EMP24::HIS3 ERV25::HIS3 ERP1::HIS3 ERP2::HIS3*	This study

medium (20 ml) containing 0.1% glucose for 1 h to induce invertase synthesis. The cells were then collected by centrifugation, washed twice with ice-cold sodium azide (10 mM), and resuspended in 0.1 ml of 1.4 M sorbitol, 50 mM Tris-HCl, pH 7.5, and 40 mM β-mercaptoethanol containing 50 U of lyticase. After 30 min incubation at 30°C, spheroplasts were sedimented at 3000 × *g* for 10 min and lysed by resuspension in 50 mM Tris-HCl, pH 7.5, and 0.1% Triton X-100. Samples corresponding to 1 OD_{600} unit of cells were electrophoresed on 8% nondenaturing polyacrylamide gels at 4°C. Invertase was localized in gels after incubation in 0.1 M sodium acetate, pH 5.1, containing 0.1 M sucrose for 30–60 min at 30°C as described (Grossmann and Zimmermann, 1979).

Preparation of Protein Samples, Electrophoresis, and Immunoblotting

To assay Gas1p transport, cells were grown to midlogarithmic phase, and 2 OD_{600} units were spun down and resuspended in 100 μl of Laemmli sample buffer containing 5 mM β-mercaptoethanol. Glass beads were added, and the cells were lysed in a bead beater (Biospec Products, Bartlesville, OK). Lysates were boiled and loaded onto 8% SDS-polyacrylamide gels. After transfer to nitrocellulose membranes, blots were incubated with primary and secondary antibodies, and bands were detected using ECL (Amersham Pharmacia Biotech, Uppsala, Sweden). Gas1p antiserum (provided by H. Riezman, University of Basel, Basel, Switzerland) was used at a 1:30,000 dilution. Goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) was used at a 1:15,000 dilution.

To monitor Kar2p secretion, exponentially growing cells were washed, resuspended in fresh medium, and incubated at 30°C. Samples (0.3 OD_{600} units of cells) were taken at t = 0, 2, and 4 h (see Figure 4) or at 4.5 h (see Figure 6) and centrifuged for 5 min. Proteins from cell supernatants were precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 10% with the addition of 5 μg of ovalbumin as carrier and to serve as a standard to monitor transfer efficiency. Kar2p was detected by SDS-PAGE (4–20% gels) and Western blotting with an anti-Kar2p antibody

(1:10,000 dilution; kindly provided by Mark Rose, Princeton University, Princeton, NJ).

Protein extracts for the analysis of Emp24p, Erv25p, Erp1p, and Erp2p were prepared from 10 OD_{600} units of a log phase culture resuspended in 300 μl of 10% TCA. Cells were lysed by vigorous agitation in the presence of glass beads. Crude extracts were spun at maximum speed in a microcentrifuge, and the resulting pellets were washed with cold acetone. Pellets were resuspended in 200 μl of Laemmli sample buffer containing 5 mM β-mercaptoethanol, and proteins were resolved on 12% polyacrylamide gels. Antisera to Emp24p (provided by H. Riezman) and Erv25p (provided by C. Barlowe, Dartmouth Medical School, Hanover, NH) were used at 1:5000 and 1:2000 dilutions, respectively. Antiporin mAb 16G9-E6 (Molecular Probes, Eugene, OR) was used at 0.5 μg/ml.

Erp1p antibodies were raised in rabbits to a C-terminal peptide (CQMKHLGKFFVKQKIL). Peptide was coupled via the N-terminal cysteine residue to keyhole limpet hemacyanin using maleimide (SMCC; Pierce, Rockford, IL) as the cross-linker. An initial subcutaneous injection of an emulsion of peptide in complete Freund's adjuvant was followed by four boosts of peptide (100 μg each) in incomplete adjuvant. Erp1p antiserum was used at a 1:1000 dilution. The Erp1 antiserum was affinity purified by conjugating peptide to Sulfolink coupling gel (6% cross-linked agarose column containing immobilized iodacetyl groups; Pierce). Any remaining sites were blocked with 50 mM cysteine in 50 mM Tris and 5 mM EDTA, pH 8.5. Elution was with 0.1 M glycine, pH 2.5. Fractions were collected and neutralized with 1 M Tris, pH 8.0. Antibodies against Erp2p were raised in rabbits to an N-terminal peptide (SKECLYYDMVT-EDDC, amino acids 40–53) and were used at a 1:500 dilution.

Radiolabeling and Immunoprecipitation

Cells were grown to midlogarithmic phase in minimal medium with 2% glucose. Cell samples (5 OD_{600} units) were collected by centrifugation, washed once with medium, and resuspended in 1 ml of fresh minimal medium. After 10 min at 30°C, 100 μCi of Trans ^{35}S label (ICN Biochemicals, Costa Mesa, CA) were added, and cells were incubated for 5 min. The chase period was initiated by adding 120 μl of 10× chase solution (50 mM methionine and 10 mM cysteine) to a 1× final concentration. At the indicated time points, labeled samples of 1 OD_{600} unit of cells were collected and chilled on ice in the presence of 20 mM NaN_3 and 20 mM NaF. Cell lysis with glass beads and immunoprecipitation of Gas1p were performed as described (Horvath *et al.*, 1994). The samples were resolved on 8% SDS-polyacrylamide gels. The gels were fixed, dried, and visualized in a Molecular Dynamics (Sunnyvale, CA) PhosphorImager.

For precipitation of Erp1 protein complexes cells were grown to midlogarithmic phase in YEPD medium. Approximately 100 OD units of cells were harvested, washed one time in PBS, and resuspended in 1 ml of ice-cold RIPA assay (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 8.0) containing protease inhibitors (Complete; Boehringer Mannheim, Mannheim, Germany). The cells were lysed in the presence of glass beads (0.5 mm) by vortexing vigorously seven times for 1 min in 50-ml tubes. The lysates were centrifuged at 500 × *g* to remove unbroken cells, and the supernatant (~900 μl) was transferred to 1.5-ml Eppendorf tubes. Triton X-100 was added to a 1% final concentration, and the samples were incubated on ice for 45 min. The lysates were then agitated for 20 min at 4°C with 50 μl of protein A-Sepharose beads (Amersham Pharmacia Biotech). After this preclearing step, 5 μl of affinity-purified anti-Erp1p antibody (4 mg/ml) was added, and the samples were incubated overnight at 4°C with end-over-end rotation. Protein A-Sepharose beads (30 μl) were added, and the samples were agitated for an additional 1 h at 4°C. The immune complexes were collected by centrifugation, washed three times with TNET (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, and 1% Triton X-100), one time in TNE (TNET without Triton X-100), and dissociated with 40 μl of twofold concentrated SDS sample buffer. One-fifth of each sample per lane was separated by SDS-PAGE. To quantify the levels of Erp1p, Erp2p, Emp24p, and Erv25p in the immunoprecipitates, equal amounts of starting material, immunoprecipitation fraction, and depleted fraction were analyzed by Western blotting with antibodies against the individual p24 proteins followed by densitometric analysis.

Gel Filtration Chromatography

Yeast cells were converted to spheroplasts as described (Rexach *et al.*, 1994) and lysed in 4% octyl glucoside, 100 mM NaCl, 10% glycerol, 2 mM PMSF, and 20 mM HEPES, pH 7.5, at a protein concentration of 5 mg/ml. The extract was cleared by centrifugation (100,000 × *g* for 30 min at 4°C), and 200 μl of the resulting supernatant were applied to a Superose 6 HR 10/30 gel filtration column (Amersham Pharmacia Biotech). The run was performed in 1% octyl glucoside, 100 mM NaCl, 10% glycerol, and 20 mM HEPES, pH 7.5, at a flow rate of 0.25 ml/min, and 500-μl fractions were collected. The fractionation was calibrated by immunoblotting against protein complexes of known size and by a parallel run of molecular weight markers (Amersham Pharmacia Biotech).

Accession Numbers

The p24 genes in this study have the following GenBank accession numbers: *EMP24*, 1322830; *ERV25*, 854478; *ERP1* (YAR002CA), 1339991; *ERP2* (YAL007C), 349746; *ERP3* (YDL018C), 1430986; *ERP4* (YOR016C), 1420117; *ERP5* (YHR110W), 529120; and *ERP6* (YGL002W), 1322449.

RESULTS

The Yeast p24 Gene Family Contains Eight Nonessential Genes

Comparison of Emp24p and Erv25p with the complete yeast genome sequence revealed six ORFs coding for putative proteins which share overall structural and sequence similarity with Emp24p and Erv25p (Figure 1A). Each predicted protein contains a hydrophobic region that might serve as an N-terminal signal sequence and is probably cleaved at the positions indicated. Near the predicted C terminus of each predicted protein there is a further stretch of hydrophobic residues capable of spanning the lipid bilayer. It is highly likely therefore that, like *EMP24* and *ERV25*, these new genes encode type I transmembrane proteins with short (10- to 12-amino acid) cytoplasmically exposed C termini and larger N-terminal lumenal domains. The yeast genome sequence is complete, and therefore this is the first case in which the complete complement of p24 proteins in one organism has been identified. We have named these new p24 genes *ERP1* (YAR002CA), *ERP2* (YAL007C), *ERP3* (YDL018C), *ERP4* (YOR016C), *ERP5* (YHR110W), and *ERP6* (YGL002W) for Emp24p- and Erv25p-related proteins. The similarity of each of the eight yeast p24 proteins to those previously identified in other species is shown in Figure 1B.

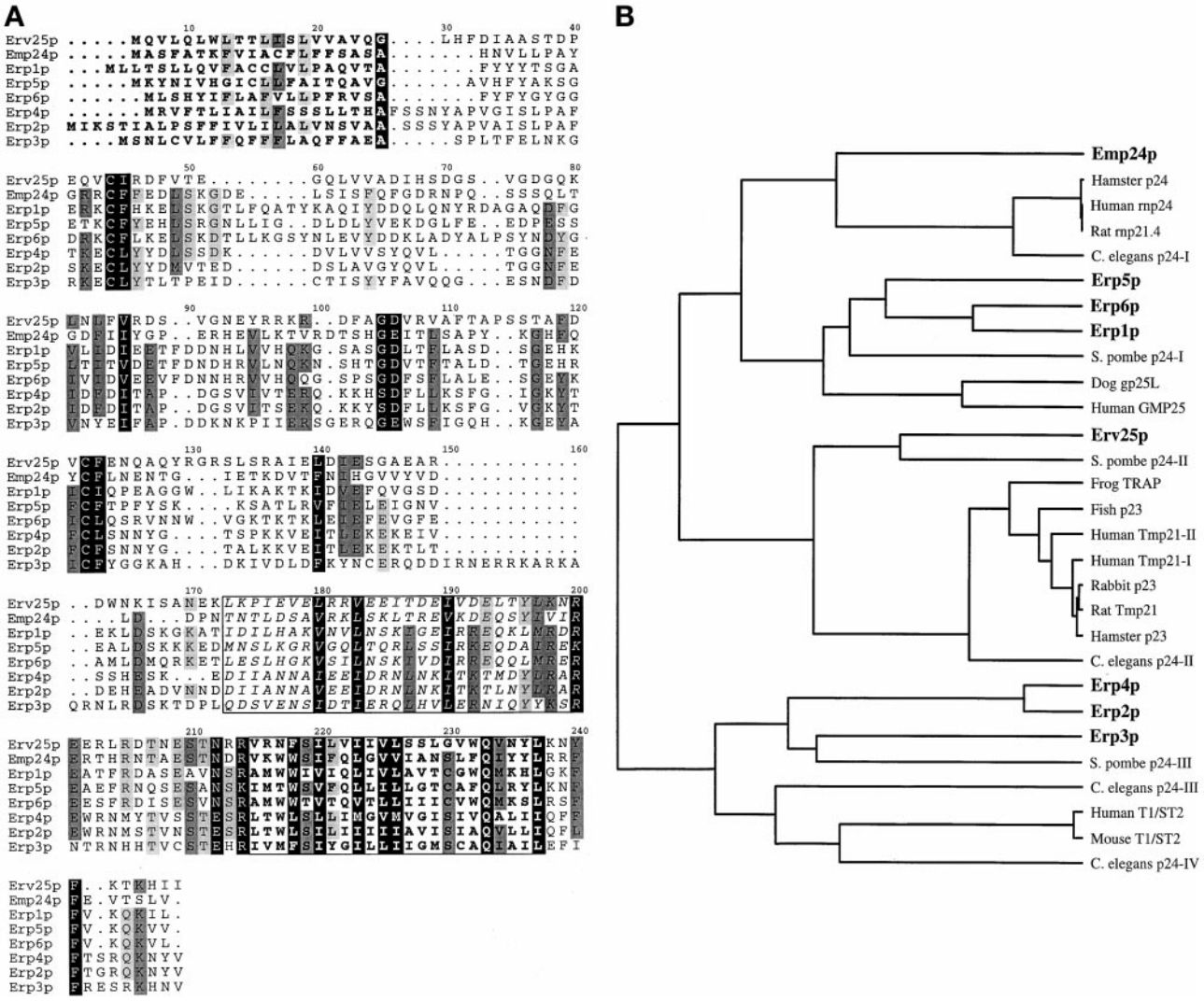

Figure 1. Yeast p24 proteins and the relationship of currently identified p24 genes. (A) Alignment of the eight p24 members in *S. cerevisiae*. The predicted N-terminal signal sequences are marked in bold. The putative coiled coil domains are boxed, and in italics and the predicted transmembrane region is boxed and in bold. All yeast p24 proteins, as in other species, contain two conserved cysteine residues within the N-terminal region of the protein that is likely to be in the lumen of a secretory compartment or vesicle. Each yeast p24 protein contains a conserved glutamine residue within the transmembrane domain and a conserved phenylalanine residue (implicated in COPII binding; Dominguez *et al.* 1998) in the membrane-proximal region of the short cytoplasmic C-terminus. Yeast α family p24 proteins (Erp1p, Erp5p, and Erp6p) contain close matches to the COPI binding motifs KKXX and KXKXX (Cosson and Letourneur, 1994). All the yeast α and β family members (Erp1p, Erp5p, Erp6p, and Emp24p) contain a polar glutamine residue within the transmembrane domain (position 222). This residue in Chop24 has been implicated in ER localization (Fiedler and Rothman, 1997). (B) Relationship between predicted p24 protein sequences currently deposited in public domain databases. The phylogenetic tree shown was generated using the UPMGA clustering method of NEIGHBOR from the PHYLIP package (Felsenstein, 1989), the distance matrix being calculated using PROTDIST.

To characterize the function of the eight yeast p24 proteins, we individually replaced each of the p24 genes with a *HIS3* marker by a PCR-mediated disruption protocol. The resulting deletion strains were all viable and displayed growth that was indistinguishable from the isogenic wild-type strain YPH274 at all temperatures tested (14, 20, 30, and 37°C).

Protein Transport Defects in p24 Mutants

Previous studies have determined that mutations in *EMP24* and *ERV25* result in selective cargo transport defects of anterograde cargo proteins, including Gas1p and invertase (Schimmöller *et al.*, 1995; Belden and Barlowe, 1996). These results suggested a role for the Emp24p and Erv25p proteins in selective ER-to-

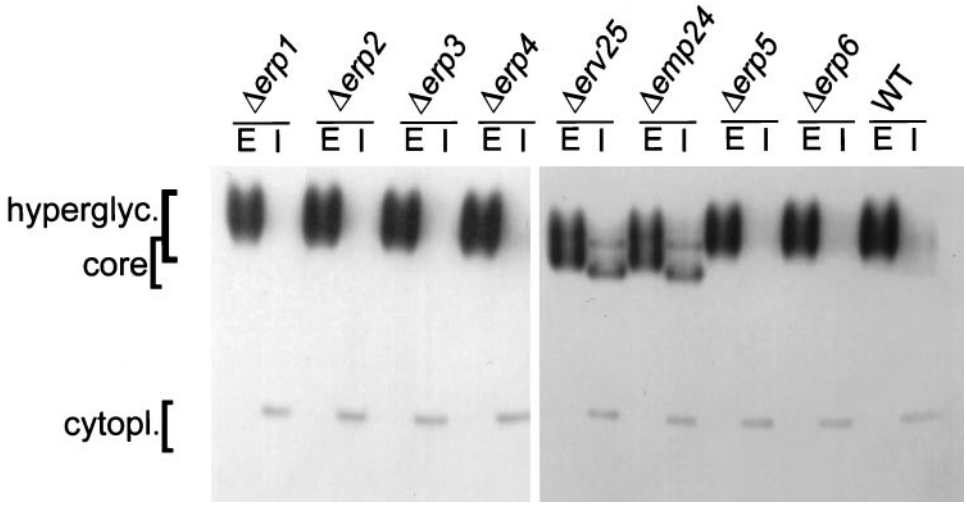

Figure 2. Invertase secretion in wild-type and p24 mutant cells. Exponentially dividing cells were transferred to low-glucose media for 1 h to induce invertase synthesis. Spheroplasts were prepared and pelleted by centrifugation to obtain the cytoplasmic (I) and periplasmic (E) fractions. Invertase was detected by an in-gel activity assay. The cytoplasmic, core, and hyperglycosylated forms of invertase are indicated.

Golgi transport. To examine the potential role of the six additional p24-related proteins in protein trafficking, we first assessed transport of invertase (Figure 2). Invertase is synthesized and is modified by the addition of N-linked oligosaccharides in the ER. Upon transport to the Golgi, invertase is further modified by extensive elongation of these carbohydrate side chains to generate a heterogeneous 100- to 150-kDa population of hyperglycosylated molecules. This latter form of invertase is rapidly secreted into the periplasmic space. To assay invertase secretion, cells were incubated in low-glucose YEP medium at 30°C to derepress invertase synthesis. Cells were converted to spheroplasts and then separated by centrifugation into internal and external fractions. Samples of both fractions were subjected to nondenaturing gel electrophoresis, and the gels were stained for invertase activity. In the *EMP24::HIS3* deletion strain (DHY3) we observed a significant accumulation of core glycosylated invertase in the internal fraction, in contrast to wild-type cells (DHY9), in which little invertase was present in the internal fraction (Figure 2). In addition, the mature invertase in the external fraction was hypoglycosylated (indicated by increased electrophoretic mobility) relative to invertase in the external fraction in wild-type cells. This result is consistent with earlier pulse–chase analysis that indicated both delayed invertase secretion and underglycosylation of secreted invertase in a strain deleted for *EMP24* (Schimmöller *et al.*, 1995). An identical observation was made for cells carrying a deletion of the *ERV25* gene. This new finding is consistent with previous data demonstrating a physical interaction between these two gene products in COPII-coated vesicles (Belden and Barlowe, 1996). Using this assay we observed no internal accumulation or hypoglycosylation of secreted invertase in any of the six additional p24 deletion strains. It is therefore unlikely that these additional six members, individually at least, are required for efficient invertase transport.

To assess the transport of the GPI-anchored protein Gas1p we first monitored the accumulation of Gas1p precursor by Western blot analysis. Gas1p is synthesized as a 105-kDa GPI-anchored precursor containing *N*- and *O*- linked core oligosaccharides that is modified in the Golgi to generate a 125-kDa mature protein. Figure 3A demonstrates that, as expected from earlier analysis, *EMP24::HIS3* (DHY3) and *ERV25*::*HIS3* (DHY4) deletion strains contain significantly more 105-kDa Gas1p precursor than wild-type cells (DHY9). Precursor accumulation was slightly more pronounced in the case of the *emp24* deletion strain than in the *erv25* deletion strain. In addition, strains deleted for two additional p24 genes, *ERP1* (DHY8) and *ERP2* (DHY7), exhibited an accumulation of Gas1p precursor (Figure 3A). To assess this phenotype more carefully in the case of DHY8 (*ERP1::HIS3*), we analyzed Gas1p transport by pulse–chase analysis. Cells were pulse labeled with ^{35}S-Cys/Met for 5 min and then chased with an excess of unlabeled amino acids. Like *emp24*- and *erv25*-null strains, the *erp1* deletion strain exhibited a significant delay in Gas1p maturation (Figure 3B). This result suggests that proteins encoded by genes *ERP1* and *ERP2* play a role similar to Emp24p and Erv25p in Gas1p transport.

Members of the p24 family have been postulated to encode cargo receptors or adaptors for ER-to-Golgi transport (Schimmöller *et al.*, 1995; Stamnes *et al.*, 1995; Belden and Barlowe, 1996). We therefore considered the possibility that p24 family members not affected in Gas1p or invertase secretion might be involved in transport of, for example, carboxypeptidase Y (CPY) or the pheromone precursor prepro-α-factor. To obtain a measure of any defect in CPY transport we looked for accumulation of CPY precursor in p24 deletion strains by Western blot analysis. In all p24

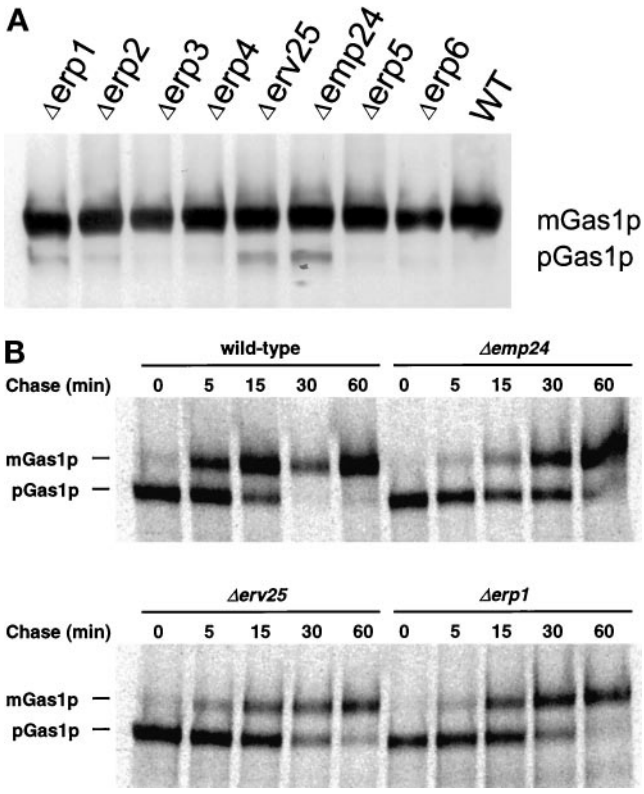

Figure 3. Maturation of Gas1p in wild-type and p24 deletion strains. (A) Whole-cell extracts were prepared from exponentially growing cells, separated by SDS-PAGE, and detected by Western blotting using an anti-Gas1p antibody. Migration of mature (mGas1p) and precursor (pGas1p) forms of Gas1p are indicated. (B) Extracts were prepared from wild-type, Δ*emp24*, Δ*erv25*, and Δ*erp1* cells after a 5-min pulse with ^{35}S-labeled amino acids followed by chase for the indicated times. Gas1p was precipitated from the extracts, and immune complexes were precipitated, separated by SDS-PAGE, and detected by autoradiography. Migration of mature (mGas1p) and precursor (pGas1p) forms of Gas1p is indicated.

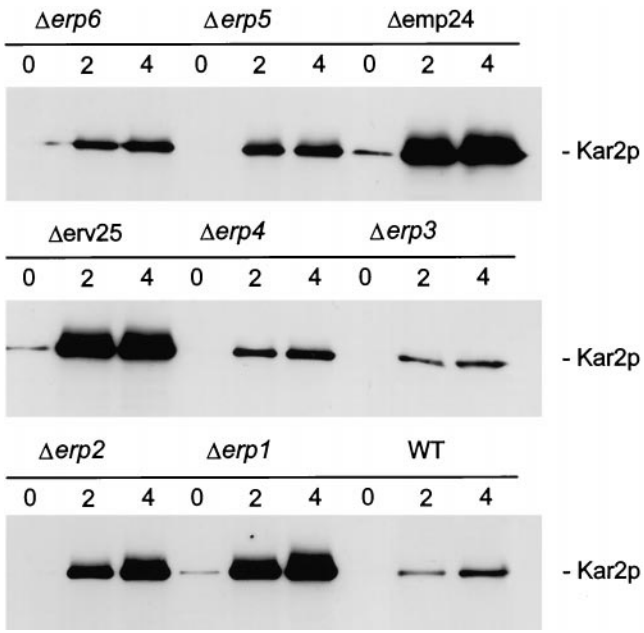

Figure 4. Analysis of Kar2p secretion in p24 mutant strains. Exponentially dividing cells were washed and resuspended in fresh medium. Proteins of the culture medium of 0.3 OD_{600} unit equivalents were TCA precipitated at the indicated times and resolved by SDS-PAGE. Kar2p was detected by Western blotting with anti-Kar2p antiserum.

mutants CPY was processed to the mature form, and no accumulation of the precursor was observed (our unpublished results). This result argues that transport of CPY in p24 deletion strains is normal, although by this analysis we cannot exclude the possibility of very minor delays. Similarly, pulse–chase analysis has revealed no defect in α-factor transport in any of the p24 mutants constructed so far (our unpublished results).

Kar2p Secretion in p24 Mutants

Previous analysis has demonstrated that deletion of *EMP24* results in the secretion of Kar2p (BiP) into the culture supernatant (Elrod-Erickson and Kaiser, 1996). To determine whether a deletion of any other p24 gene might result in a similar phenotype, we washed cells growing in exponential cultures with fresh medium to remove previously secreted Kar2p, and then proteins secreted into the culture supernatant after 2 and 4 h of incubation in fresh medium were TCA-precipitated before detecting Kar2p by Western blotting. Figure 4 shows a representative experiment demonstrating that, like deletion of *EMP24*, deletion of *ERV25* results in significant levels of Kar2p secretion into the culture supernatant. Densitometric analysis of these data indicates that after a 4-h incubation in fresh medium, *emp24* mutants secrete at least 15 times more BiP, and *erv25* mutants secrete at least 14 times more BiP than wild-type cells. Two additional mutants show marked increases in the amount of BiP secretion into the growth medium; a strain deleted for *ERP1* (DHY8) secretes at least 12 times more Kar2p, and an *erp2* deletion strain (DHY7) secretes at least nine times more BiP than wild-type cells in the same experiment (Figure 4). Remaining deletion strains all secrete more BiP than wild-type cells, but the differences are less marked (all less than fivefold more BiP than wild-type cells). All mutants contained identical amounts of intracellular Kar2p as determined by Western blot analysis of total cell extracts. These results further suggest that Erp1p and Erp2p share a similar function with Emp24p and Erv25p.

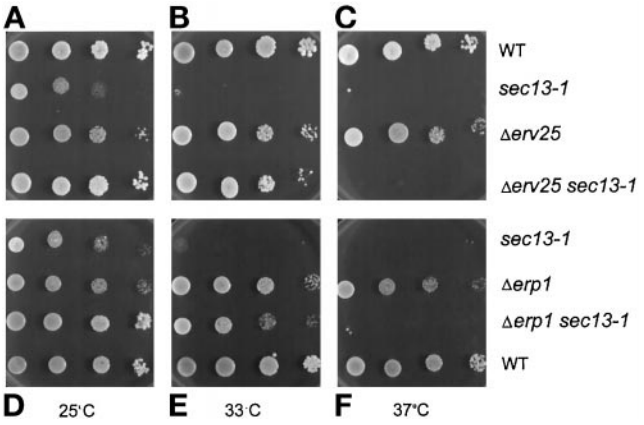

Figure 5. Suppression of *sec13-1* by p24 deletion mutations. Each p24 deletion mutant was crossed with a *sec13-1* temperature-sensitive mutant, and segregants from a tetratype tetrad were tested for growth at different temperatures on YEPD medium. Results shown are at 25°C, a permissive *sec13-1* temperature (A and D), 33°C (B and E), and 37°C (C and F), nonpermissive temperatures for *sec13-1* mutants.

Genetic Interactions of p24 Mutations

Mutations in *EMP24* have previously been identified as bypass of *SEC13* deletion (*bst1*) mutations (Elrod-Erickson and Kaiser, 1996); Δ*sec13 bst1* double mutants are viable, whereas *sec13*-null mutants are otherwise inviable. We tested the ability of deletion mutations in each of *ERP1–ERP6*, as well as in *ERV25*, to suppress a temperature-sensitive *sec13-1* mutation. A cross between a *sec13-1* temperature-sensitive strain and strains bearing mutations in either *ERP1–ERP6* or *ERV25* generated four viable spore products in all cases when spore germination was perfomed at 23°C. The ability of all segregants from at least two tetratype tetrads to grow at temperatures of 25, 30, 31, 32, 33, 34, 35, 36, and 37°C was tested by spotting 5 μl of 10-fold serial dilutions from a log phase culture onto YEPD plates. In this experiment *emp24 sec13-1* double mutants were able to grow at temperatures up to 33°C, whereas a *sec13-1* mutant was unable to grow at temperatures >30°C. Figure 5 demonstrates that mutations in two additional genes, *ERV25* and *ERP1*, produced a similar phenotype in combination with a *sec13-1* temperature-sensitive mutation. Both *sec13-1 erv25::HIS3* and *sec13-1 erp1::HIS1* strains grew at temperatures up to 33°C, whereas *sec13-1* segregants from the same tetrad were unable to grow at temperatures >30°C. In all other cases (strains deleted for *ERP2*, *ERP3*, *ERP4*, *ERP5*, or *ERP6*) we observed no *sec13-1* suppression at any temperature.

One explanation for the lack of identifiable phenotypes in four of the p24 deletion strains is that there is functional redundancy among family members (Figure 1B). To address this possibility we created double mutants in all of the most closely related pairs of p24 genes (all pair-wise combinations of *ERP1*, *ERP5*, and *ERP6* and all pair-wise combinations of *ERP2*, *ERP3*, and *ERP4*). The identity between these gene pairs ranges from 70% in the case of *ERP2* and *ERP4* to 28% in the case of *ERP3* and *ERP2*. In all cases these double mutants were viable and exhibited no significant growth defects. When we examined secretion of Kar2p in these double mutants we found no evidence of additive phenotypes; each double mutant secreted the same amount of Kar2p as the most severely affected single mutant. Double mutants, furthermore, showed no evidence of delays in CPY transport or α-factor secretion (our unpublished results). To test this still further, we constructed triple mutants of *ERP1*, *ERP5*, and *ERP6* (ARY101) and *ERP2*, *ERP3*, and *ERP4* (ARY102). These comprise all the yeast members of the α and β/γ families, respectively, according to the terminology recently suggested by Dominguez *et al.* (1998) (also see Figure 1B). As before, no growth defects were observed in these triple mutants, and no defects in CPY and α-factor secretion were detected (our unpublished results). Furthermore, we detected no additive effects on Kar2p or Gas1p secretion.

Deletions in three genes, *EMP24*, *ERV25*, and *ERP1*, exhibit similar phenotypes with respect to Gas1p transport, Kar2p secretion, and *sec13-1* suppression. These genes are not the most closely related at the amino acid sequence level; for example, Emp24p and Erv25p are the most closely related and are only 26% identical. Nevertheless, it remains possible that these gene products play redundant roles in transport. To test this idea and to examine the possibility that deletions in these genes might reveal synthetic interactions, we constructed a triple *erp1*, *emp24*, and *erv25* deletion strain (ARY103). This strain was viable and exhibited no growth defects or further defects in protein transport. Deletion of *ERP2* also results in similar defects in Gas1p and Kar2p transport. We also therefore constructed a strain (ARY104) containing disruptions of all four genes (*EMP24*, *ERV25*, *ERP1*, and *ERP2*) with similar defects in Gas1p and Kar2p transport. This strain was also viable and displayed no growth defects or additive effect on the Kar2p secretion phenotype.

To take a different approach we tested whether moderate overexpression of related p24 genes from the TPI promotor might complement the Kar2p secretion defect of *erp1* and *erp2* mutants. TPI-p24 constructs were individually introduced into *erp1* and *erp2* mutants, and the Kar2p secreted over a 4.5-h incubation was measured by Western blot analysis. Growth rates of all strains were identical, and these levels of p24 overexpression did not lead to an increase in Kar2p secretion in wild-type cells. The results shown in Figure 6 demonstrate that in both cases, introduction of the wild-type gene lowered the amount of Kar2p secreted by *erp1* and *erp2* mutant

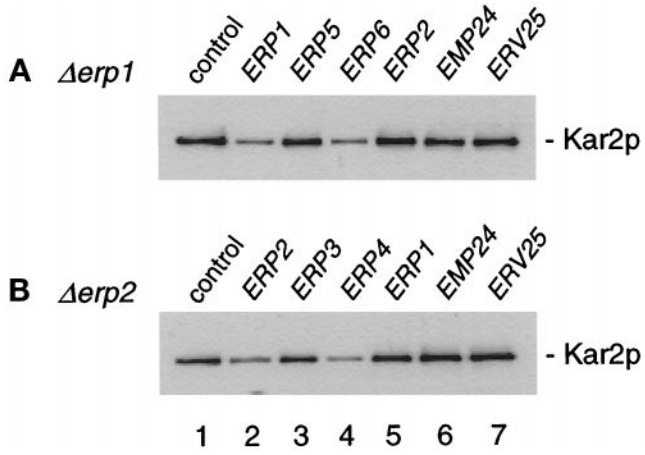

Figure 6. Genetic redundancy among p24 genes. Individual p24 genes were overexpressed from the *TPI* promotor in *erp1* or *erp2* mutant cells grown at 30°C in YPD. Exponentially dividing cells were washed and resuspended in fresh medium. Proteins of the culture medium of 0.3 OD_{600} unit equivalents were TCA precipitated after 4.5 h and resolved by SDS-PAGE. Kar2p was detected by Western blotting with anti-Kar2p antiserum.

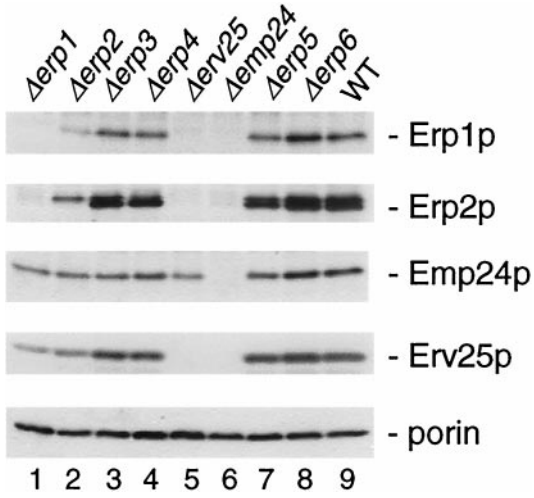

Figure 7. Emp24p, Erv25p, Erp1p, and Erp2p protein levels are interdependent. Total cell extracts from wild-type and p24 mutant cells were separated by SDS-PAGE, and proteins were detected on Western blots using Emp24p, Erv25p, Erp1p, or Erp2p antisera as indicated. A mAb to porin, a mitochondrial membrane protein unrelated to this study, was used as a loading control.

cells as expected (Figure 6, compare lanes 1 and 2). The amount of Kar2p secreted by these complemented mutants was indistinguishable from that secreted by wild-type cells. Normal Kar2p secretion in *erp1* mutants was restored by both *ERP1* and *ERP6*, whereas the defect in *erp2* mutants was suppressed by both *ERP2* and *ERP4*. Overexpression of *EMP24* or *ERV25* had no effect on the level of Kar2p secreted by either mutant. *ERP6* is very closely related to *ERP1* (58% identical), whereas *ERP4* is closely related to *ERP2* (70% identical) (Figure 1B). These results demonstrate that the function of *ERP1* can be substituted by elevated levels of *ERP6* and that *ERP2* function can be substituted by *ERP4*, at least as measured by Kar2p secretion. This is the first evidence of functional redundancy among p24 proteins.

p24 Protein Levels Are Interdependent

The biochemical and genetic experiments described above suggest that Emp24p, Erv25p, Erp1p, and Erp2p function in a cooperative manner. We therefore considered the possibility that these four proteins may interact in a protein complex. This idea is consistent with earlier results demonstrating that Erv25p and Emp24p can be cross-linked together in COPII-coated vesicles and that the stability of these proteins is interdependent (Belden and Barlowe, 1996). To address the question of whether Erp1p and Erp2p interact with Emp24p or Erv25p at a biochemical level, we examined the levels of Emp24p and Erv25p in *emp24*, *erv25*, *erp1*, and *erp2* deletion strains. As expected, Emp24p was not detected in an *emp24* deletion strain (DHY3); similarly, strains carrying an *ERV25* deletion contained no Erv25 protein (DHY4) (Figure 7, lanes 5 and 6). Consistent with earlier analyses, Erv25 protein levels were significantly lower, in our experiments almost undetectable, in a strain deleted for *EMP24* than in wild-type cells (Figure 7, compare lanes 6 and 9). Previous analysis has demonstrated that this is due to a decrease in Erv25p stability in the absence of Emp24p (Belden and Barlowe, 1996). Also consistent is our observation of a threefold decrease in Emp24p protein level in an *erv25* deletion strain (Figure 7, lane 5). Significantly, both Emp24p and Erv25p levels were lower in strains deleted for *ERP1* (DHY8) or *ERP2* (DHY7) than in wild-type cells (Figure 7, lanes 1 and 2). Densitometric analysis of these data suggests that Emp24p and Erv25p levels are approximately threefold and sixfold lower, respectively, in the *ERP1::HIS3* deletion strain than in wild-type cells (Figure 7, lane 1). These results therefore suggest that Emp24p and Erv25p stability may be dependent not only on the presence of each other but also on Erp1p and Erp2p. To test this idea further, we raised Erp1p antibodies and examined Erp1 protein levels in strains deleted for *EMP24* and *ERV25* and in five strains deleted for the additional p24 genes identified in this study. Figure 7 demonstrates that, as expected, no Erp1 protein is detected in an *ERP1::HIS3* strain (Figure 7, lane 1). Significantly, the levels of Erp1p in strains deleted either for *EMP24* or *ERV25* are greatly diminished (virtually undetectable) relative to wild-type and strains deleted for any of the five remaining p24 genes (Figure 7, compare lanes 5 and 6 with lane 9). These results strongly suggest that the stability of Erp1p

depends on the presence of Emp24p and Erv25p and that these three gene products may function together in a complex.

Analysis of Erp1 protein levels in the additional p24 deletion strains revealed that Erp1p levels were significantly reduced in a further p24 deletion strain; in a strain containing an *ERP2::HIS3* deletion (DHY7) the level of Erp1 protein was almost 10-fold lower than in the isogenic wild-type strain (Figure 7, lane 2). The *erp2* mutant also exhibited lower levels of Emp24p (twofold lower) and Erv25p (threefold lower). These observations suggest that Erp2p may also interact with Emp24p, Erv25p, and Erp1p, although the reduction in Erp1p, Erv25p, and Emp24p protein levels is less than what we have observed in other cases. We have already noted above, however, that there is significant sequence similarity between p24 family members such that functional redundancy might mask interactions by the approach we have taken (Figure 7). To address this possibility we examined Emp24p, Erv25p, and Erp1p levels in an *erp2*, *erp3*, *erp4* triple mutant (β/γ family; Dominguez *et al*., 1998; also see Figure 1B). However, we could find no further decrease in Emp24, Erv25, and Erp1 protein levels over that seen in the single *erp2* deletion strain. To study this further we looked at the level of the Erp2 protein in the p24 deletion mutants. Strikingly, Erp2p was almost undetectable in strains deleted for *ERP1*, *EMP24*, or *ERV25* (Figure 7, lanes 1, 5, and 6), indicating that the stability of the Erp2 protein is dependent on the presence of Erp1p, Emp24p, and Erv25p. These results again suggest that these four proteins function together in a heteromeric complex. No significant differences in the levels of Emp24p, Erv25p, or Erp1p were observed in strains deleted for any of the additional four p24 genes (*ERP3–6*) (Figure 7).

Erp1p and Erp2p Interact with Emp24p and Erv25p in a Heteromeric p24 Complex

The genetic and biochemical evidence described above demonstrates that four p24 genes, *EMP24*, *ERV25*, *ERP1*, and *ERP2*, can be mutated to give similar phenotypes and that the levels of the p24 proteins encoded by these four genes are interdependent. One explanation for these observations might be that these four p24 proteins interact with one another in a heteromeric p24 protein complex. To test this idea we immunoprecipitated Erp1p from wild-type and p24 mutant cells. Figure 8A demonstrates that Erp1p was detected in immunoprecipitates from a wild-type strain but not from a mutant deleted for the *ERP1* gene (Figure 8A, compare lanes 1 and 4). Quantitative immunoblot analysis of the immunoprecipitate and of the cell extract before and after immunoprecipitation demonstrated that, under these conditions, >90% of the Erp1p in the extract was immunoprecipitated (Figure 8B). No Erp1p was detected in immunoprecipitates with preimmune serum or unrelated antibodies. No Erp1p was detected in immunoprecipitates from strains deleted for Emp24p (Figure 8A, lane 3), consistent with the Western blot analysis of Erp1p levels in *EMP24*-deleted cells (Figure 7). We then determined the presence of other p24 proteins in Erp1p immunoprecipitates. Emp24p, Erv25p, and Erp2p were all detected in anti-Erp1p immunoprecipitates prepared from wild-type cells (Figure 8A, lanes 5, 9, and 13), whereas two unrelated membrane proteins, Dpm1p and porin, were not detected. Quantitative immunoprecipitation experiments in which cell extracts were compared before and after immunoprecipitation revealed that ~30% of the Emp24p was depleted by incubation with the Erp1p antibody (Figure 8B). For Erv25p the amount depleted was ~29%, and for Erp2p it was ~34%. In each case the amount of p24 protein depleted was broadly consistent with the amount recovered in the immunoprecipitate (Figure 8B). As expected, Emp24p was not detected in anti-Erp1p immunoprecipitates prepared from an *EMP24::HIS3* strain (Figure 8A, lane 7). We have demonstrated that in this strain Erp1p is present at reduced levels (Figure 7). Consistent with this observation, Erv25p and Erp2p were also not detected in anti-Erp1p immunoprecipitates prepared from the *EMP24::HIS3* strain, even though Erv25p at least was readily detected in whole cell extracts (Figure 7). Interestingly, Emp24p, Erv25p, and Erp2p were still detected, albeit at reduced levels, in Erp1p immunoprecipitates from cells carrying a deletion of the *ERP1* gene (Figure 8A, lanes 8, 12, and 16). To account for this observation we considered the possibility that in *ERP1::HIS* mutant cells, Emp24p, Erv25p, and Erp2p might be coimmunoprecipitating with Erp5p and/or Erp6p, both closely related to Erp1p (Figure 1). Although by Western blot analysis we did not detect significant cross-reactivity of the anti-Erp1p antiserum with Erp5/6p (Figure 7), a more-sensitive ELISA revealed that the Erp1p antiserum, affinity purified against a C-terminal Erp1p peptide, exhibits cross-reactivity with an equivalent peptide from Erp6p. To test the idea that immunoprecipitation of Emp24p, Erv25p, and Erp2p is dependent on Erp5p and Erp6p in the absence of Erp1p, we examined anti-Erp1p precipitates from cells deleted for the *ERP1*, *ERP5*, and *ERP6* genes. Figure 8A, lanes 6, 10, and 14, shows that the simultaneous deletion of these three genes resulted in immunoprecipitates devoid of Emp24p, Erv25p, and Erp2p, demonstrating that coimmunoprecipitation of these p24 proteins is dependent on Erp5p and/or Erp6p in the absence of Erp1p. This is the first demonstration of a function for Erp5p and Erp6p, a function that appears to be redundant with Erp1p, consistent with the strong sequence similarities observed between these genes (Figure 1)

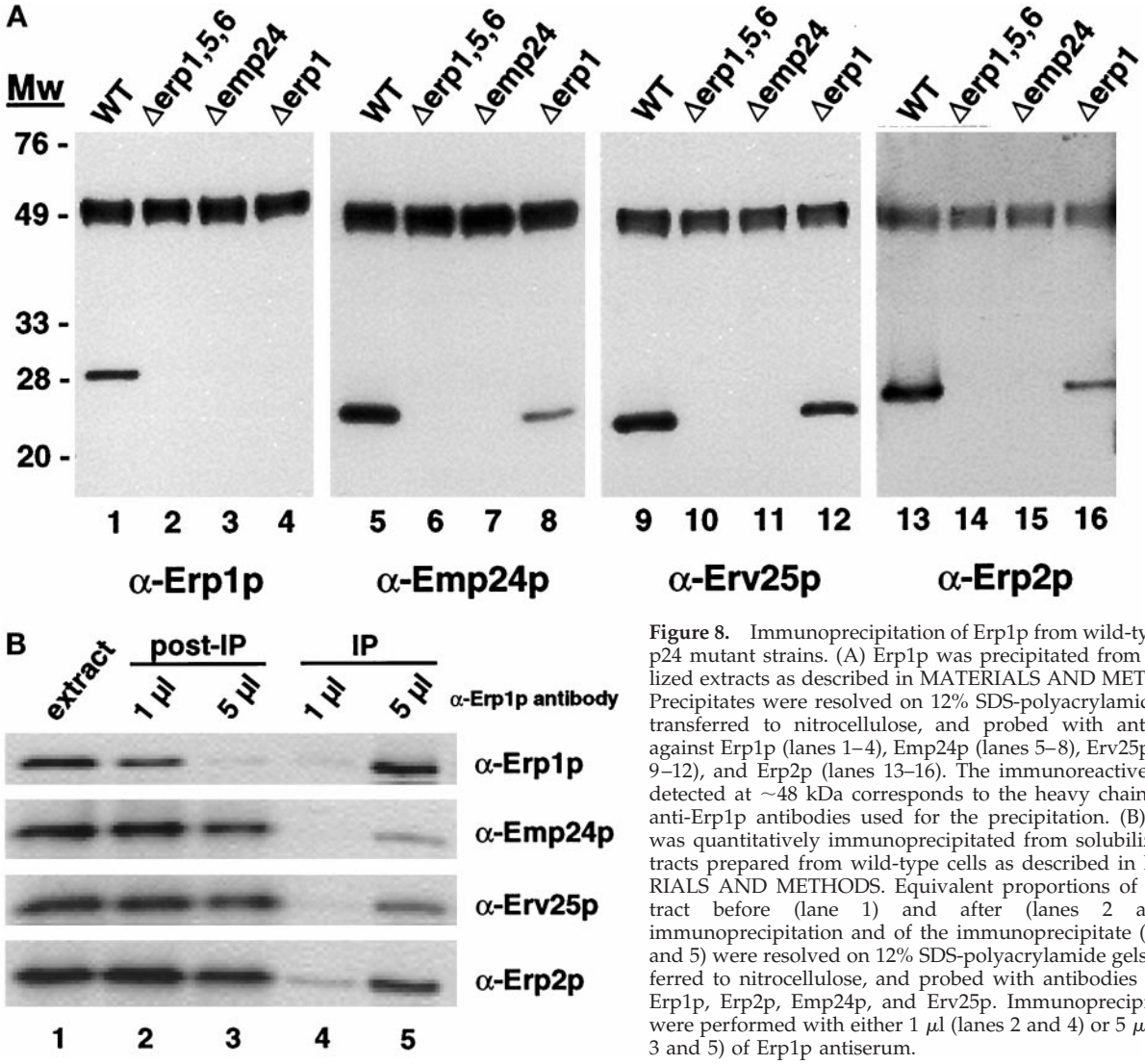

Figure 8. Immunoprecipitation of Erp1p from wild-type and p24 mutant strains. (A) Erp1p was precipitated from solubilized extracts as described in MATERIALS AND METHODS. Precipitates were resolved on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies against Erp1p (lanes 1–4), Emp24p (lanes 5–8), Erv25p (lanes 9–12), and Erp2p (lanes 13–16). The immunoreactive signal detected at ~48 kDa corresponds to the heavy chain of the anti-Erp1p antibodies used for the precipitation. (B) Erp1p was quantitatively immunoprecipitated from solubilized extracts prepared from wild-type cells as described in MATERIALS AND METHODS. Equivalent proportions of the extract before (lane 1) and after (lanes 2 and 3) immunoprecipitation and of the immunoprecipitate (lanes 4 and 5) were resolved on 12% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies against Erp1p, Erp2p, Emp24p, and Erv25p. Immunoprecipitations were performed with either 1 μl (lanes 2 and 4) or 5 μl (lanes 3 and 5) of Erp1p antiserum.

and the ability of *ERP6* to complement the Kar2p secretion phenotype of *erp1* mutant cells (Figure 6).

To determine the specificity of Erp1p immunoprecipitations we prepared whole cell extracts from cells metabolically labeled with ^{35}S-methionine and -cysteine. Erp1p immunoprecipitates were prepared from extracts from both wild-type cells and from a strain deleted for the *ERP1*, *ERP5*, and *ERP6* genes. Figure 9A shows that the only proteins that are specifically immunoprecipitated by the Erp1p antiserum migrate in the 24- to 30-kDa range. Comparison of the migration of these proteins with the migration of Emp24p, Erv25p, Erp1p, and Erp2p in a parallel experiment in which immunoprecipitates were prepared from unlabeled cells and p24 proteins detected by Western blotting strongly suggests that the immunoprecipitated radiolabeled proteins correspond to these same four p24 proteins (Figure 9B). In addition, mass spectrometry analysis of Coomassie Blue–stained gel bands from the Erp1p immunoprecipitates unambigously identified the same four p24 proteins (our unpublished results). These data demonstrate that under the conditions we have used the coimmunoprecipitatation of Emp24p, Erv25p, and Erp2p with Erp1p is completely specific.

The results described above strongly suggest that Erp1p is found in a complex (or complexes) with Emp24p, Erv25p, and Erp2p. To determine whether these four proteins are found in the same complex and to determine the size of p24-containing complexes in yeast, we performed gel filtration separation of yeast extracts followed by Western blot analysis of the fractions with p24 antibodies (Figure 10). A significant proportion of Erp1p, Erp2p, Emp24p, and Erv25p

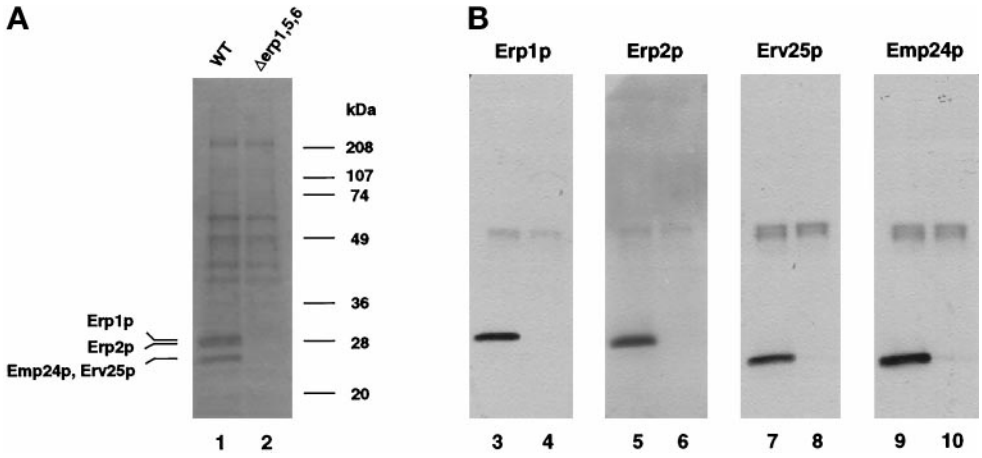

Figure 9. Immunoprecipitation of Erp1p from radiolabeled wild-type and *erp1/5/6* mutant cells. Erp1p was precipitated from solubilized extracts prepared from radiolabeled wild-type or *erp1/5/6* mutant cells (A) or unlabeled cells (B) as described in MATERIALS AND METHODS. Immunoprecipitates were resolved on 12% SDS-polyacrylamide gels and either dried and exposed to film (A) or transferred to nitrocellulose (B) and probed with antibodies against Erp1p (lanes 3 and 4), Erp2p (lanes 5 and 6), Erv25p (lanes 7 and 8), and Emp24p (lanes 9 and 10). Immunoprecipitates in lanes 1, 3, 5, 7, and 9 were prepared from wild-type cells; those in lanes 2, 4, 6, 8, and 10 were prepared from *erp1/5/6* mutant cells. The immunoreactive protein detected at ~48 kDa corresponds to the heavy chain of the anti-Erp1p antibodies used for the immunoprecipitation.

from wild-type cell extracts were found to coelute at a molecular mass of ~100 kDa, consistent with these four p24 proteins forming a heteromeric complex, as suggested by the biochemical and genetic experiments described above (Figure 10A). Each of the four p24 proteins also eluted at lower-molecular-mass positions, and it is therefore possible that p24 proteins exist in lower-molecular-mass complexes or that, under these in vitro conditions at least, p24 complexes are unstable. Only a minority of each p24 protein eluted from the column in fractions that would be expected to contain p24 monomers. Interestingly, when extracts were prepared from *erp1/erp5/erp6* triple deletion mutants, Emp24p and Erv25p continued to coelute from the gel filtration column but in later fractions, most consistent with p24 dimers (Figure 10B). In these cells the amount of Emp24p and Erv25p recovered was also reduced relative to wild-type cells, consistent with earlier results (Figure 7). Erp2p was previously shown to be undetectable in these extracts (Figure 7). This result demonstrates that the presence of Emp24p and Erv25p in higher-molecular-mass p24 complexes is dependent on the presence of Erp1p and/or Erp2p. Furthermore, in the absence of Erp1p (resulting indirectly in the absence of Erp2p) most Emp24p and Erv25p is present in p24 dimers.

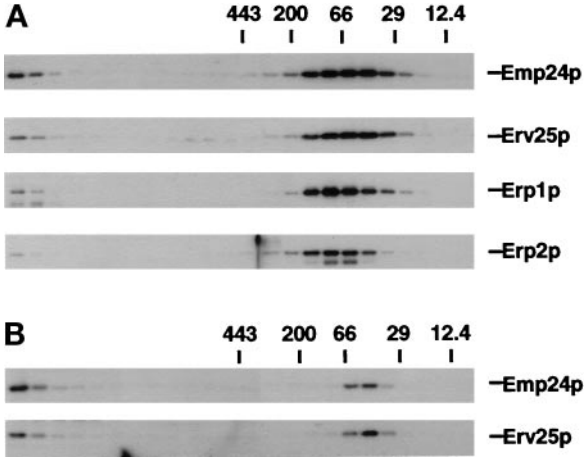

Figure 10. Gel filtration chromatography. Wild-type (A) and *erp1/erp5/erp6* mutant (B) yeast cells were converted to spheroplasts and lysed, and the cleared extract was applied to a Superose 6 HR 10/30 gel filtration column. The fractionation was calibrated by parallel runs of molecular weight markers. Individual p24 proteins were detected by Western blot analysis.

DISCUSSION

In this study we have identified six additional members of the p24 family in the budding yeast *S. cerevisiae*. These are in addition to two previously identified p24 genes, *EMP24* and *ERV25*, and we have therefore named these new genes *ERP1–ERP6* for Emp24p- and Erv25p-related proteins. Because the genome of this organism has been sequenced in its entirety, this system affords the first opportunity to study the complete complement of p24 proteins in a eukaryotic cell.

All yeast p24 proteins are predicted to exhibit type I structural organization similar to one another and to p24 proteins identified in other species (Figure 1; Wada *et al.*, 1991; Holthuis *et al.*, 1995; Schimmöller *et al.*, 1995; Stamnes *et al.*, 1995; Belden and Barlowe, 1996; Blum *et al.*, 1996; Fiedler *et al.*, 1996; Sohn *et al.*, 1996; Rojo *et al.*, 1997; Dominguez *et al.*, 1998). The eight p24 proteins identified in *S. cerevisiae* is the highest number found in any one species to date. The predicted p24 proteins encoded by the six new ORFs fall into two groups based on comparison of amino acid sequences (Figure 1). Comparison of these sequences with those of other species demonstrates that

Erp1p, Erp5p, and Erp6p are members of the p24 α family, and Erp2p, Erp3p, and Erp4p are members of the β/γ family, according to nomenclature recently proposed by Dominguez *et al.* (1998) (Figure 1B). Emp24p and Erv25p are the sole *S. cerevisiae* members of the β and δ families, respectively. The sequence and structural similarities between p24 proteins from yeast and other species suggest that the functions of these proteins have been conserved through evolution.

We have demonstrated that none of the eight p24 genes is essential for viability in budding yeast, and all deletion mutants grow at rates equivalent to wild type, consistent with earlier observations for *EMP24* and *ERV25* (Schimmöller *et al.*, 1995; Belden and Barlowe, 1996). Nevertheless, deletion of *EMP24*, *ERV25, ERP1*, or *ERP2* results in a selective transport defect of Gas1p, a GPI-anchored cell surface protein (Figure 3). Deletion of these same four genes also leads to increased secretion of the ER resident protein Kar2p into the culture supernatant (Figure 4). A further phenotype previously ascribed to *emp24* deletion mutants is shared by *erv25* and *erp1* deletion mutants; all three mutant genes suppress, to equal extents, a temperature-sensitive mutation in *SEC13*, a COPII vesicle coat protein gene (Figure 5). Interestingly, we detected no defect in invertase transport in *erp1* or *erp2* mutants, unlike in *emp24* (Schimmöller *et al.*, 1995) and *erv25* mutants (Figure 2). Although not identical, the similarity among *emp24*, *erv25*, *erp1*, and *erp2* mutant phenotypes led us to conclude that these four gene products are likely to function, perhaps together, in the early secretory pathway.

Because of the close sequence similarity of *ERP1*, *ERP5*, and *ERP6* and of *ERP2*, *ERP3*, and *ERP4*, we considered the possibility of functional redundancy. However, by combining deletions we were unable to demonstrate any additive phenotypic effects, suggesting that if *ERP5*, *ERP6, ERP3*, and *ERP4* have a function that is redundant with that of *ERP1* and *ERP2*, they contribute only a minor proportion of the total p24 protein in the cell. This would be consistent with the minor Kar2p secretion defects exhibited by *erp5*, *erp6*, *erp3*, and *erp4* mutants (Figure 4) and the lack of any other phenotype, although it remains possible that one or more of these genes may be required for an activity that we have not measured or that is only required under certain physiological conditions. Nevertheless, by moderately overexpressing *ERP4* (70% identical to *ERP2*) or *ERP6* (58% identical to *ERP1*), we demonstrated that these two genes, like the relevant wild-type genes, but not *EMP24* or *ERV25*, are capable of restoring normal Kar2p secretion to *erp2* or *erp1* mutant cells, respectively (Figure 6). Taken together these results suggest that *ERP4* shares a redundant function with *ERP2*, and *ERP6* is redundant with *ERP1*, but that in vivo it is predominantly *ERP1* and *ERP2* that fulfill these roles.

To address the possibility that Emp24p, Erv25p, Erp1p, and Erp2p function together at the same step of the secretory pathway, we first asked whether the levels of all four of these proteins are interdependent. Both Erp1p and Erp2p levels were almost undetectable in *emp24* and *erv25* deletion mutants, demonstrating that the steady-state levels of these proteins are dependent on both Emp24p and Erv25p (Figure 7). Consistent with this, Erv25p levels were also significantly lower in the *emp24* deletion, as previously described (Belden and Barlowe, 1996), as well as in the *erp1* and *erp2* deletion strains. In the case of Erv25p, the decrease in protein level in an *emp24* mutant was shown to be a consequence of a decrease in Erv25p protein stability and that Emp24p and Erv25p interact with one another in COPII-coated vesicles (Belden and Barlowe, 1996). The simplest explanation for our observations is that Erp1p and Erp2p protein levels are dependent on the presence of Emp24p and Erv25p, because a significant proportion of these four p24 proteins function together in one or more heteromeric p24 complexes.

To further investigate the possibility that Emp24p, Erv25p, Erp1p, and Erp2p interact, we immunoprecipitated Erp1p from wild-type and p24 mutant cells (Figures 8 and 9). Emp24p, Erv25p, and Erp2p specifically coimmunoprecipitated with Erp1p. These observations strongly suggest that Emp24p, Erv25p, Erp1p, and Erp2p function interdependently in a p24 protein complex. The strong interdependence of p24 protein levels suggests that, particularly in the case of Erp1p and Erp2p, which are both present at very low levels in the absence of Emp24p and Erv25p, a significant proportion of p24 protein in the cell is likely to be present in p24 complex(es). This idea is further supported by gel filtration chromatography of yeast extracts during which Erp1p, Erp2p, Erv25p, and Emp24p elute from the column at greater than their molecular mass, strongly supporting the idea that they exist in multimeric complexes in vivo. Significantly, there is a peak in the elution of all four p24 proteins at a molecular mass of ~100 kDa, consistent with the combined molecular mass of Erp1p, Erp2p, Emp24p, and Erv25p and strongly supporting the hypothesis that these four proteins interact in vivo (Figure 10). Furthermore, gel filtration experiments using *erp1/erp5/erp6* mutant cells demonstrate that the presence of Emp24p and Erv25p in higher-molecular-mass p24 complexes is dependent on the presence of Erp1p and/or Erp2p. In the absence of Erp1p (resulting indirectly in the absence of Erp2p), most remaining Emp24p and Erv25p is present in p24 dimers. If these dimers have at least some residual functionality, this would explain why deletion of *ERP1* and or *ERP2* is less deleterious than deletion of *EMP24* and/or *ERV25* (Figures 2–4).

Erp1p is most similar to the mammalian p24 proteins GMP25 and gp25L (α1 and α2; Dominguez *et al.*, 1998) (Figure 1B). Both gp25L (α1) and GMP25 (α2) contain a C-terminal sequence (KKLV) that matches a KKXX consensus binding motif for COPI binding. Indeed, GMP25 (α2) has recently been demonstrated to strongly bind COPI subunits in vitro (Dominguez *et al.*, 1998). Similarly, Erp1p contains a potential COPI binding site (KQKIL) and binds COPI subunits very efficiently (Rowley, Dejgaard, and Nilsson, unpublished observations). Interestingly, mutation of the KK motif in GMP25 (α2) both reduced COPI binding in vitro and resulted in the redistribution of GMP25 (α2) from the *cis*-Golgi network to later compartments of the secretory pathway, including the cell surface, in transiently transfected cells (Dominguez *et al.*, 1998). Remarkably, when this mutant GMP25 (α2) was cotransfected with four additional (nonmutated) p24 proteins (p23 [δ1], p24 [β1], gp27 [γ1], and p26 [γ2]) these too were found to redistribute to later secretory compartments, suggesting that mammalian p24 proteins are interdependent for their appropriate cellular localization. This observation was supported by the observation that these same p24 proteins cofractionate when purified from rat liver Golgi membranes. It is interesting to note that Emp24p, Erv25p, and Erp2p are most similar to mammalian p24 (β1), p23 (δ1), and gp27 (γ1) or p26 (γ2), respectively; therefore both in yeast and mammalian cells p24 members of the α, β, δ, and γ sequence families appear to interact. The parallels between our findings in yeast and those of Dominguez *et al.* (1998) in mammalian cells suggest that the ability of p24 proteins to form heteromeric complexes has been conserved throughout evolution. In this respect it is interesting to note that in other model organisms with almost sequenced genomes (*Schizosaccharomyces pombe* and *Caenorhabditis elegans*) these same p24 proteins appear to be represented in most cases by a single member (Figure 1B), suggesting that these four p24 proteins may represent a core p24 complex.

The role of p24 proteins in membrane traffic is still unclear. Evidence in both yeast and mammalian cells suggests that cargo molecules are actively packaged into COPII-coated vesicles (Schekman and Orci, 1996), and p24 proteins have previously been suggested as candidates for cargo receptors or adaptors (Schimmöller *et al.*, 1995; Stamnes *et al.*, 1995). The effects on invertase (*emp24* and *erv25* mutants) and Gas1p (*emp24*, *erv25*, *erp1*, and *erp2*) transport observed by Schimmöller *et al.* (1995), Belden and Barlowe (1996), and ourselves are still consistent with such a role. Nevertheless, we have not yet identified, as predicted by this cargo receptor model, additional transport defects that would suggest that p24 proteins play a role in the selective concentration of cargo molecules such as CPY and α-factor into COPII-coated vesicles, although we cannot exclude the possibility of very minor defects below the level of detection in our experiments or that we have not yet deleted the critical combination of p24 genes. Recently, experiments from the Riezman laboratory have suggested the existence of two parallel transport pathways from the ER to the Golgi in yeast (Sütterlin *et al.*, 1997). It remains possible that the p24 proteins identified here as playing a role in Gas1p transport are only required for transport mediated by one of these two pathways and that another family of "transport factors" is required for incorporation of cargo molecules such as α-factor and CPY into a second class of COPII-coated vesicle.

Several lines of evidence suggest that p24 proteins could play a role in retrograde transport from the Golgi to the ER. This role might involve a function in COPI vesicle formation or structure or perhaps a role as a cargo receptor, adaptor, transport factor. First, in both yeast and mammalian cells the C-terminal tails of at least a subset of p24 proteins have been shown to interact with subunits of the COPI vesicle coat (Fiedler *et al.*, 1996; Sohn *et al.*, 1996; Dominguez *et al.*, 1998) and may be enriched in COPI-coated vesicles, although there is conflicting evidence from different studies (Rojo *et al.*, 1997). Second, deletion of p24 proteins in yeast results in secretion of elevated levels of the ER resident protein Kar2p (Figure 4; Elrod-Erickson and Kaiser, 1996), suggesting that the Erd2p-dependent retrieval process may be defective in these cells. This possibility is strengthened by our recent finding that secretion of an HDEL-tagged version of pro-α factor in a strain simultaneously deleted for *EMP24*, *ERV25*, *ERP1*, and *ERP2* is as fast as that of endogenous α factor, whereas HDEL-α factor is held back in the early secretory pathway when expressed in a wild-type background (our unpublished observations). The delay observed in Gas1p and invertase anterograde transport does not exclude a role for p24 proteins in retrograde traffic, because a number of studies in both yeast and mammalian cells have demonstrated that defects in retrograde transport can lead to indirect effects on anterograde transport. For example, in yeast a number of genes that are now known to encode subunits of the COPI coat complex, and which exhibit complete blocks in retrograde transport when mutant, were first identified by mutations that result in anterograde transport defects (Hosobuchi *et al.*, 1992; Duden *et al.*, 1994). Recently, Gaynor and Emr (1997) have isolated new temperature-sensitive alleles of *SEC21* that encode the γ subunit of the yeast COPI protein complex. In addition to a complete block in retrograde transport, cells bearing these new alleles display cargo-selective α factor and CPY anterograde transport defects, whereas the transport of other cargo molecules, such as invertase, proceeds normally. Recent experiments have demonstrated a Gas1p anterograde transport defect in several COPI mutants, a

phenotype that was particularly pronounced in a *ret1-1* mutant (Sütterlin *et al.*, 1997). *RET1* encodes the α-COP subunit of coatomer. The similarity between this phenotype and the delay that we observe in *emp24*, *erv25*, *erp1*, and *erp2* mutants could be explained in two ways. *EMP24*, *ERV25*, *ERP1*, and *ERP2* could encode rate-limiting Gas1p transport factors that fail to be recovered to the ER in a *ret1-1* cell, resulting in an indirect delay in anterograde Gas1p transport. Alternatively, Emp24p, Erv25p, Erp1p, and Erp2p, like Ret1p, could be more directly involved in the retrograde transport process. At present it is not possible to distinguish between these possibilities. It is interesting to note that although both *sec21* and *ret1-1* mutants do not show a defect in invertase transport, both mutations result in a general decrease in the glycosylation state of invertase (Gaynor and Emr, 1997; Sütterlin *et al.*, 1997). The invertase secreted by *emp24* and *erv25* mutants cells is also hypoglycosylated (Schimmöller *et al.*, 1995; Figure 2), suggesting that all these mutants may share a common underlying defect in function within the early Golgi.

In conclusion, although the function of p24 proteins remains to be determined, we have demonstrated that four different yeast p24 proteins, Emp24p, Erv25p, Erp1p, and Erp2p, can be mutated to give similar phenotypes and that these four proteins interact in a p24 protein complex. Our results suggest that heteromeric p24 complexes may be a conserved feature of p24 function in all eukaryotic cells.

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