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Deletion of Yeast p24 Genes Activates the Unfolded Protein Response

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Yeast cells lacking a functional p24 complex accumulate a subset of secretory proteins in the endoplasmic reticulum (ER) and increase the extracellular secretion of HDEL-containing ER residents such as Kar2p/BiP. We report that a loss of p24 function causes activation of the unfolded protein response (UPR) and leads to increased *KAR2* expression. The HDEL receptor (Erd2p) is functional and traffics in p24 deletion strains as in wild-type strains, however the capacity of the retrieval pathway is exceeded. Other conditions that activate the UPR and elevate *KAR2* expression also lead to extracellular secretion of Kar2p. Using an in vitro assay that reconstitutes budding from the ER, we detect elevated levels of Kar2p in ER-derived vesicles from p24 deletion strains and from wild-type strains with an activated UPR. Silencing the UPR by *IRE1* deletion diminished Kar2p secretion under these conditions. We suggest that activation of the UPR plays a major role in extracellular secretion of Kar2p.

INTRODUCTION

In eukaryotic cells, secretory proteins and lipids are synthesized at the endoplasmic reticulum (ER) and then transported to intracellular organelles or the plasma membrane via the secretory pathway (reviewed by Kaiser *et al.*, 1997). Several lines of evidence indicate protein sorting occurs during export from the ER such that anterograde secretory cargo is selected for export in comparison to ER resident proteins (Balch *et al.*, 1994; Rexach *et al.*, 1994; Bednarek *et al.*, 1995). Current models suggest a coat protein complex, termed COPII, mediates this sorting event by forming vesicles and including the desired set of cargo molecules by direct or indirect interaction (Springer *et al.*, 1999). In addition to coat-dependent selection into vesicles, mechanisms of ER retention (Sato *et al.*, 1996) and retrieval (Semenza *et al.*, 1990) operate to maintain overall compartmental organization of the secretory pathway.

A family of transmembrane proteins, known as the p24 proteins, form heteromeric complexes and influence sorting during transport through the early secretory pathway. Initially discovered as abundant proteins contained on ER membranes (Wada *et al.*, 1991), and then identified on COPI vesicles (Stamnes *et al.*, 1995) and COPII vesicles (Schimmoller *et al.*, 1995), the p24 proteins have been proposed to function as cargo receptors (Schimmoller *et al.*, 1995; Muñiz *et al.*, 2000), as negative regulators of vesicle budding (Elrod-Erickson and Kaiser, 1996), or as structural components of vesicles (Bremser *et al.*, 1999), ER (Lavoie *et al.*, 1999), and Golgi (Rojo *et al.*, 2000). Deletion of all eight p24 genes in yeast produces viable cells that display phenotypes (Springer *et al.*, 2000) exhibited by the single deletion of *EMP24* (Schimmoller *et al.*, 1995) or *ERV25* (Belden and Barlowe, 1996). These single deletions appear to destabilize heteromeric p24 complexes, leading to a general loss of p24 function (Marzioch *et al.*, 1999). Under this loss of function condition, the secretory proteins Gas1p and invertase are transported at reduced rates and partially accumulate in the ER. In contrast, ER resident proteins that contain an HDEL retrieval sequence (e.g., Kar2/BiP) escape the early secretory pathway and are secreted into the extracellular medium. In addition, loss of p24 function suppresses a deletion of *SEC13*, an essential gene that encodes a subunit of the COPII vesicle coat (Elrod-Erickson and Kaiser, 1996). Although some of these phenotypes seem consistent with current models for p24 function (Kaiser, 2000), others are less clear. In this report we examine mechanisms underlying secretion of Kar2p to provide insight into the role of p24 proteins in membrane trafficking. We find that extracellular secretion of Kar2p involves the induction of a stress response pathway.

An intracellular signaling pathway known as the unfolded protein response (UPR) controls ER homeostasis and protein folding in eukaryotic cells (reviewed by Chapman *et al.*, 1998). In yeast, the UPR transcriptionally regulates >300 genes, including ER-resident chaperones such as Kar2p, Pdi1p (protein disulfide isomerase), Fkb2p (peptidy-prolyl *cis*-trans isomerase), and a PDI-like protein encoded by *EUG1* (Sidrauski *et al.*, 1998; Travers *et al.*, 2000). The UPR also regulates ER lipid synthesis by negatively affecting the Opi1p repressor of lipid synthesis (Cox *et al.*, 1997). The UPR is activated when unfolded proteins accumulate in the ER and this can be triggered experimentally by treating cells

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with compounds that interfere with protein folding in the ER (e.g., tunicamycin and β-mercaptoethanol). An ER-localized kinase, Ire1p, is thought to somehow sense the accumulation of unfolded proteins in the ER and then acts as a specific endoribonuclease in splicing the *HAC1* mRNA. The translation product of spliced *HAC1* mRNA then acts as a transcriptional activator for a set of genes that contain an upstream UPR element (UPRE), including the *KAR2* gene (Sidrauski and Walter, 1997). In this report, we find that deletion of p24 genes leads to activation of the UPR and that secretion of Kar2p is due in large part to activation of this pathway.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions

Yeast strains used in this study were grown in rich media (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose) or selective media (0.67% yeast nitrogen base without amino acids, 2% dextrose, and required supplements). These growth conditions and other standard genetic methods used have been described (Sherman, 1991). When indicated, cultures were treated with 15 mM β-mercaptoethanol to activate the UPR (Cox and Walter, 1996). The optical densities of cell cultures were measured at 600 nm in a Beckman DU40 model spectrophotometer.

Strain Construction

All strains used in this report are listed in Table 1. Strains expressing a c-myc–tagged version of Erd2p were generated by transformation with the plasmid pJS209 (Semenza *et al.*, 1990). An isogenic set of strains containing the *ire1*Δ allele was made by repeated backcrosses of MS3548 (Beh and Rose, 1995) with FY834 and then CBY114 or CBY99 (Belden and Barlowe, 1996). Strains with the UPRE-*LacZ* reporter construct were generated by transformation with pJC31 (Cox and Walter, 1996). Overexpression of *KAR2* was achieved by transformation with a 2 μ plasmid containing the *KAR2* gene (pMR109) as previously described (Rose *et al.*, 1989).

Antibodies and Immunoblotting

Antibodies specific for Kar2p (Brodsky *et al.*, 1993), Sec61p (Stirling *et al.*, 1992), Erv25p (Belden and Barlowe, 1996), Emp24p (Schimmoller *et al.*, 1995), Bos1p (Cao and Barlowe, 2000), Emp47p (Schroder *et al.*, 1995), Gas1p (Frankhauser and Conzelmann, 1991), Gdi1p (Garrett *et al.*, 1994), and c-myc (Evan *et al.*, 1985) were used in this study at dilutions previously described. Protein samples were electophoretically separated on 12.5% polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose membranes for immunoblotting (Towbin *et al.*, 1979). Primary antibodies bound to nitrocellulose were detected using horseradish peroxidase-conjugated secondary antibody followed by chemiluminescence (Amersham-Pharmacia, Piscataway, NJ).

Kar2p Secretion

Extracellular Kar2p secretion was analyzed as previously described (Elrod-Erickson and Kaiser, 1996; Marzioch *et al.*, 1999) with minor modifications. Stationary phase cultures were back diluted into rich media and grown to mid-logarithmic phase. Logarithmic stage cells were then harvested, washed, and resuspended in fresh rich medium at equivalent cell densities (OD_{600} = 0.5). After growth for 1 and 3 h, 1.5 ml of the cultures was centrifuged at 14,000 × *g* for 5 min and 1.35 ml of the supernatant fluids was collected. Proteins contained in this extracellular media were precipitated by adding 0.15 ml of 100% trichloroacetic acid (TCA) (Sigma Chemical, St Louis, MO) and incubated on ice for 20 min. The precipitated proteins were collected by centrifugation at 14,000 × *g* for 15 min at

Table 1. Strains used in this study

Strain	Genotype	Reference
FY833	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63*	Winston *et al.* (1995)
FY834	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63*	Winston *et al.* (1995)
CBY99	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 emp24Δ::LEU2*	Belden and Barlowe (1996)
CBY112	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 emp24Δ::LEU2 erv25Δ::HIS3*	Belden and Barlowe (1996)
CBY114	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 erv25Δ::HIS3*	Belden and Barlowe (1996)
RSY279	*MATα his4Δ619 ura3-52 sec22-3*	Kaiser and Schekman (1990)
RSY263	*MATα ura3-52 sec12-4*	Kaiser and Schekman (1990)
MS3548	*MATα ura3-52 leu2Δ1 ade2 ire1Δ::URA3*	Beh and Rose (1995)
CBY398	FY834 with pJS209 *(2μ-URA3-TPI-ERD2-myc)*	This study
CBY419	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 erv25Δ::HIS3* with pJS209	This study
CBY423	*MATα his4-619 ura3-52 sec22-3* with pJS209	This study
CBY425	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 erv25Δ::HIS3 ire1Δ::URA3*	This study
CBY427	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 ire1Δ::URA3*	This study
CBY428	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 emp24Δ::LEU2 ire1Δ::URA3*	This study
CBY548	*MATα his3Δ200 ura3-52 trp1Δ63 sec12-4* with pJS209	This study
CBY549	*MATα his3Δ200 ura3-52 leu2Δ1 trp1Δ63 erv25Δ::HIS3* with pJS209	This study
CBY550	*MATα his3Δ200 ura3-52 leu2Δ1 trp1Δ63 erv25Δ:: HIS3 sec12-4* with pJS209	This study
CBY635	FY834 with pJC31 *(UPRE-LacZ* in pRS314)	This study
CBY636	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 emp24Δ::LEU2* with pJC31	This study
CBY637	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 erv25Δ::HIS2* with pJC31	This study
CBY748	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 sec22-3* with pJC31	This study
CBY752	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 emp24Δ::LEU2 erv25Δ::HIS3* with pJC31	This study
CBY750	FY834 with pMR109 *(2μ−URA3-KAR2)*	This study
CBY751	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 erv25Δ::HIS3* with pMR109	This study
CBY983	FY834 with pJC31 and pMR109	This study
CBY984	*MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 erv25Δ::HIS3* with pJC31 and pMR109	This study

4°C, washed with 100% acetone, dried at room temperature, and resuspended in 35 μl of SDS-PAGE sample buffer supplemented with 50 mM Tris pH 9.4. One-fifth of this sample was resolved by SDS-PAGE for immunoblots or one-half for silver staining.

Cell pellets from the above-mentioned 1.5-ml cultures were lysed in SDS-PAGE sample buffer or used to obtain whole cell membrane preparations. Briefly, cells were resuspended in 0.4 ml of lysis buffer (0.1 M sorbitol, 50 mM KOAc, 2 mM EDTA, 20 mM HEPES pH 7.5, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and vortexed in the presence of one-half volume of glass beads. The resulting lysates were subjected to a clearing spin at 5000 × *g* for 5 min to remove unlysed cells and 0.2 ml of this low-speed supernatant was transferred to a new tube and membranes were isolated by centrifugation at 100,000 × *g* in a TLA100.3 rotor (Beckman Instruments, Fullerton, CA) for 15 min. The high-speed pellet that contained whole cell membranes was resuspended in 35 μl of SDS-PAGE sample buffer and one-fifth was analyzed by immunoblot.

In Vitro Budding Assays

Vesicle budding from the ER was reproduced in vitro by incubation of microsomes (Wuestehube and Schekman, 1992) with purified COPII proteins (Sar1p, Sec23p complex, and Sec13p complex) as described (Barlowe *et al.*, 1994). Where indicated, microsomes were prepared from cells grown in the presence of 15 mM β-mercaptoethanol for 1 h before harvesting cultures. To measure incorporation of proteins into COPII vesicles, a 15-μl aliquot of the total budding reaction and 150 μl of a supernatant fluid containing budded vesicles were centrifuged at 100,000 × *g* in a TLA100.3 rotor (Beckman Instruments) to collect membranes. The resulting membrane pellets were solubilized in 30 μl of SDS-PAGE sample buffer and 10–15 μl was resolved on 12.5% polyacrylamide gels. The percentages of individual proteins (Erd2p-myc, Erv25p, Bos1p, and Sec61p) packaged into vesicles from a total reaction were determined by densitometric scanning of immunoblots. Protease protected [^{35}S]glycopro-α-factor ([^{35}S]gp-α-F) packaged into budded vesicles was measured by precipitation with Concanavaline Sepharose after posttranslational translocation of [^{35}S]-prepro-α-F into microsomes (Wuestehube and Schekman, 1992). In some experiments, [^{35}S]gp-α-F was quantified by PhosphoImager analysis (Molecular Dynamics, Sunnyvale, CA) after transfer to nitrocellulose membranes and exposure to a phosphoscreen. For measurement of Kar2p contained in COPII vesicles, budded vesicles were treated with trypsin (100 μg/ml) for 10 min on ice followed by tyrpsin inhibitor (100 μg/ml) to ensure detection of a protease-protected species.

Cell Fractionation

Membrane fractions enriched in ER (p13) and Golgi (p100) were prepared from gently lysed cells as previously described (Wooding and Pelham, 1998) with minor modifications. Cell cultures (25 ml) in logrithmic growth phase at 30°C were shifted to 37°C for 30 min to invoke temperature-sensitive blocks. Cells were harvested, resuspended in a 4 ml of spheroplast buffer (0.7 M sorbitol, 10 mM Tris-Cl pH 7.4, 0.5% glucose), treated with lytic enzyme for 10 min at 37°C, chilled on ice, and spheroplasts collected by centrifugation. Spheroplasts were resuspended in lysis buffer (0.1 M sorbitol, 50 mM KOAc, 2 mM EDTA, 20 mM HEPES pH 7.5, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and lysed with a dounce homogenizer at 4°C. Unlysed cells were cleared at 2500 × *g* for 10 min and the supernatant fraction was centrifuged at 13,000 × *g* for 10 min to generate the p13 fraction. A p100 fraction was prepared from the p13 supernatant fluid after centrifugation at 100,000 × *g* for 15 min. Pellets were resuspended in 50 μl of 2× SDS-PAGE sample buffer and 15 μl was analyzed by immunoblot.

β-Galactosidase Assays

Yeast strains containing the UPRE-*LacZ* fusion construct, pJC31, were grown overnight in selective media to maintain selection of the plasmid and then back diluted into rich media to an OD_{600} = 0.2. After 6 h of growth, cells were harvested and β-galactosidase activity was measured as previously described (Asubel *et al.*, 1997). Activity is expressed in Miller Units and SE and p values were calculated as described (Remington and Schork, 1985). Where indicated, 15 mM β-mercaptoethonol was added to cultures to activate the UPR 1 h before measuring β-galactosidase activity.

Northern Blots

Log phase cultures (25 ml) were grown in the absence or presence of 15 mM β-mercaptoethanol, harvested (OD_{600} = 0.5), washed and resuspended in 0.5 ml RNA extraction buffer (100 mM LiCl, 100 mM Tris-HCl pH 7.5, 0.1 mM EDTA), and lysed with glass beads. The RNA was extracted twice with phenol/chloroform and precipitated with 2 volumes of 100% ethanol. The RNA was resuspended in 0.05 ml of TE buffer and quantified for equal loading. DNaseI-treated RNA was separated on a 1.2% agarose, 0.625% formaldehyde gel in 1× 3-(*N*-morpholino)propanesulfonic acid running buffer. The RNA was transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH) and probed for 2 h at 65°C in Perfecthyb Plus hybridization buffer (Sigma Chemical). The membranes were washed once in low-stringency buffer (1× SSC, 0.1% SDS) and twice in high-stringency buffer (0.1× SSC, 0.1% SDS) at 65°C, and then exposed to PhosphoImager screens. Probes were labeled with [α-^{32}P]dATP (New England Nuclear, Boston, MA) by using RadPrime DNA Labeling System (Life Technologies, Grand Island, NY). DNA corresponding to *HAC1* was polymerase chain reaction amplified from pJC835 (Cox and Walter, 1996) and a 1.0-kb *Eco*RI fragment of *KAR2* was obtained from pMR109 (Rose *et al.*, 1989).

RESULTS

Erd2p Traffics Independent of p24 Proteins

Loss of p24 function in yeast results in an ER accumulation of Gas1p and invertase as well as increasing the secretion of resident ER proteins (Schimmoller *et al.*, 1995; Elrod-Erickson *et al.*, 1996; Marzioch *et al.*, 1999). We sought to examine mechanisms underlying secretion of the ER-resident protein Kar2p to provide insight into the function of p24 proteins. Kar2p and other soluble resident ER proteins are correctly localized in part because a specific retrieval system acts to return residents that have leaked out. An essential component of this retrieval system is the yeast HDEL-receptor encoded by the *ERD2* gene. Erd2p binds to HDEL sequences present on the C terminus of ER resident proteins that have trafficked to the Golgi complex and subsequently returns them to the ER in a COPI-dependent manner (reviewed by Pelham, 1998). Previous studies suggested that loss of p24 function affected an ER-retention mechanism that was independent of the HDEL-retrieval pathway (Elrod-Erickson *et al.*, 1996) although additional studies indicate mammalian p24 proteins participate in retrograde trafficking of KDEL-tagged proteins (Majoul *et al.*, 1998). Indeed, many of the phenotypes associated with p24 deletion strains could be explained if the HDEL-retrieval system was impaired. To test whether the Erd2p retrieval pathway was functioning correctly in the absence of p24 proteins, we first monitored transport of Erd2p between the ER/Golgi in an *erv25*Δ strain.

Our previous studies have shown that deletion of *EMP24* or *ERV25* does not affect the formation of COPII-coated vesicles from ER membranes in vivo and in vitro (Belden and Barlowe, 1996). Furthermore, deletion of all eight p24 family members in yeast does not appear to influence the

overall rates of COPII- or COPI-dependent budding (Springer *et al.*, 2000). Some secretory proteins, however, are not efficiently exported from the ER (Schimmoller *et al.*, 1995; Springer *et al.*, 2000) apparently due to a decreased rate of incorporation into ER-derived vesicles (Muñiz *et al.*, 2000). Erd2p may also depend on the p24 complex for efficient packaging into ER-derived vesicles therefore we directly measured Erd2p packaging in an in vitro assay that reconstitutes vesicle budding and cargo selection (Salama *et al.*, 1993; Barlowe *et al.*, 1994). Cellular membranes enriched in ER (microsomes) were isolated from wild-type and *erv25*Δ strains containing an epitope-tagged version of Erd2p (Semenza *et al.*, 1990). The efficiency of Erd2p incorporation into ER-derived vesicles was measured after addition of purified COPII proteins and collection of budded vesicles (Belden and Barlowe, 1996). As seen in Figure 1A, the amounts of Erd2p packaged into COPII vesicles from wild-type and *erv25*Δ membranes were similar. As controls, Sec61p, an ER-resident protein required for membrane translocation, was not efficiently packaged into budded vesicles, whereas Bos1p, an ER/Golgi SNARE protein required for vesicle fusion, was efficiently incorporated. Approximately 3% of the total Erd2p was incorporated into ER-derived vesicles compared with 8 and 10% of Erv25p and Bos1p respectively. The differences in these percentages likely reflect the amount of each species that resides in the ER. Microsomal membrane preparations contain ER and Golgi membranes and because a majority of Erd2p is Golgi localized (Lewis and Pelham, 1992; Townsley *et al.*, 1994), we surmise that only a small fraction of this receptor is cycling through the ER and would be available for packaging into COPII vesicles. Regardless, we detected COPII-dependent release of Erd2p and the amount of Erd2p packaged into COPII vesicles was equivalent whether the p24 complex was present or not. Therefore, we conclude that p24 proteins are not required for anterograde transport of Erd2p from the ER in COPII vesicles.

Next we tested the hypothesis that p24 proteins are required for retrograde transport of Erd2p. Again, Kar2p secretion could be explained if Erd2p failed to enter COPI vesicles. To determine whether Erd2p is efficiently recycled back to the ER in an *erv25*Δ strain, we blocked COPII-dependent export with the temperature-sensitive *sec12-4* allele to accumulate cycling proteins in the ER (Lewis and Pelham, 1996). If retrograde transport of Erd2p depended on the p24 complex, an *erv25*Δ *sec12-4* double mutant strain should not accumulate Erd2p in the ER when shifted to a nonpermissive temperature. As seen in Figure 1B, Erd2p shifted equally to the ER in both *ERV25 sec12-4* and *erv25*Δ *sec12-4* strains. In this experiment, crude ER (p13) and Golgi (p100) were isolated from cells after incubation at 37°C for 30 min. As controls, Sec61p served as an ER marker and Emp47p served as a Golgi marker that cycles between these compartments as previously demonstrated (Schroder *et al.*, 1995). In wild-type and *erv25*Δ strains, both Emp47 and Erd2p maintained a Golgi localization. However, in the presence of the *sec12-4* allele, these Golgi-localized proteins shifted to the ER fraction in a manner that was independent of *ERV25*. We conclude that the p24 complex does not function in anterograde or retrograde traffic of Erd2p.

Although Erd2p appears to be cycling correctly, it may be incapable of binding HDEL proteins in the absence of a

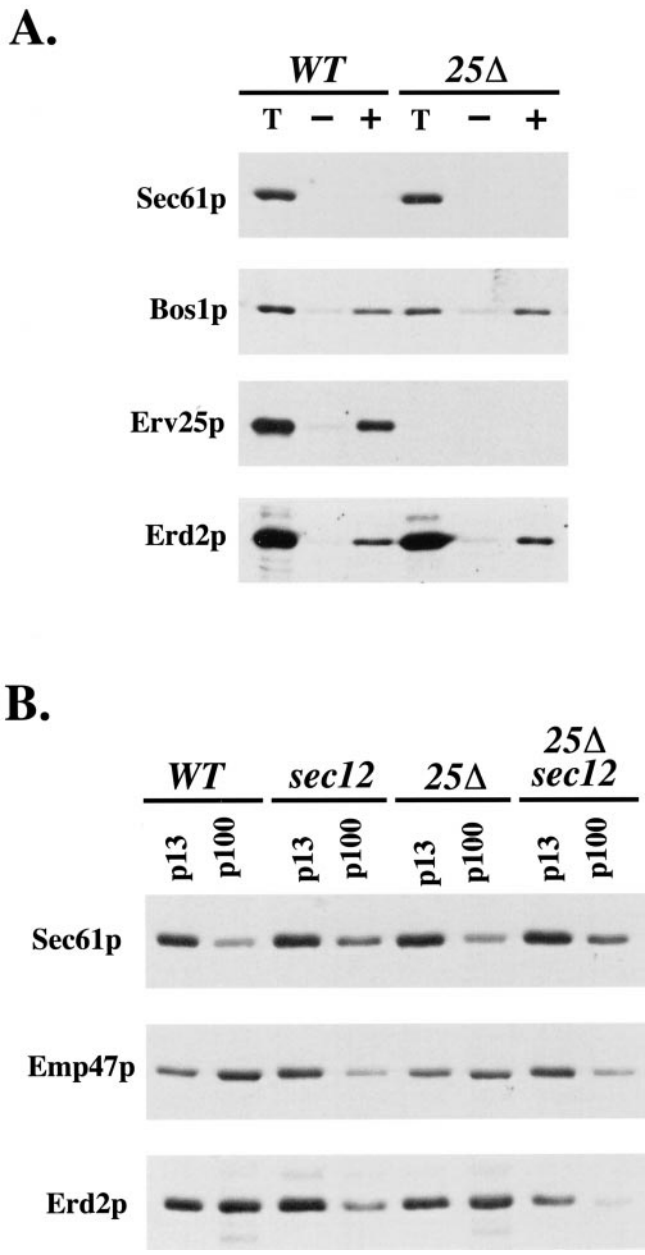

Figure 1. Erd2p transport between the ER and Golgi compartments is independent of the p24 complex. (A) Reconstituted COPII budding reactions were performed on ER membranes isolated from CBY398 (WT) and CBY419 (*erv25*Δ) strains that express an epitope-tagged version of Erd2p. Lanes labeled T represent one-tenth of the total membranes used in a budding reaction, minus (−) lanes indicate the amount of vesicles formed in the absence of the purified COPII components, and plus (+) lanes indicate vesicles produced when COPII proteins are added. Total membranes and budded vesicles were collected by centrifugation, resolved on a polyacrylamide gel, and immunoblotted for indicated proteins. (B) ER and Golgi membrane fractions from CBY398 (WT), CBY548 (*sec12*), CBY549 (*erv25*Δ), and CBY550 (*sec12, erv25*Δ) strains that express the myc-tagged version of Erd2p. Cells were shifted to 37°C for 1 h before harvesting and lysis. ER membranes were pelleted by a medium-speed centrifugation (p13) and Golgi membranes were collected by a high-speed centrifugation (p100). Proteins contained in these fractions were monitored by immunoblot analysis.

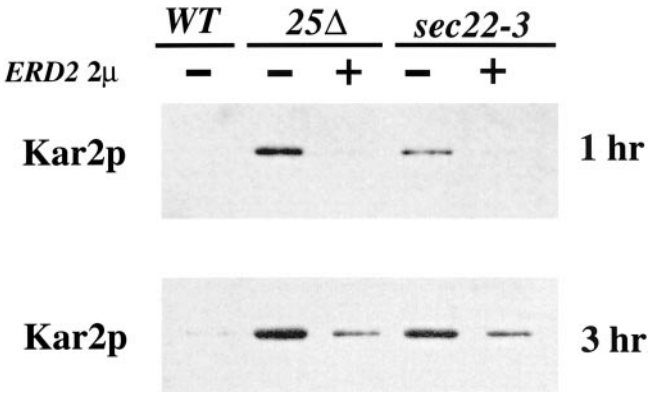

Figure 2. Overexpression of *ERD2* partially suppresses the secretion of Kar2p in an *erv25*Δ deletion strain. The amount of extracellular Kar2p secreted after 1 and 3 h in *erv25*Δ and *sec22-3* strains with and without an *ERD2* 2 μ plasmid. Proteins contained in the cell culture supernatant were concentrated by TCA precipitation and Kar2p was detected by immunoblot.

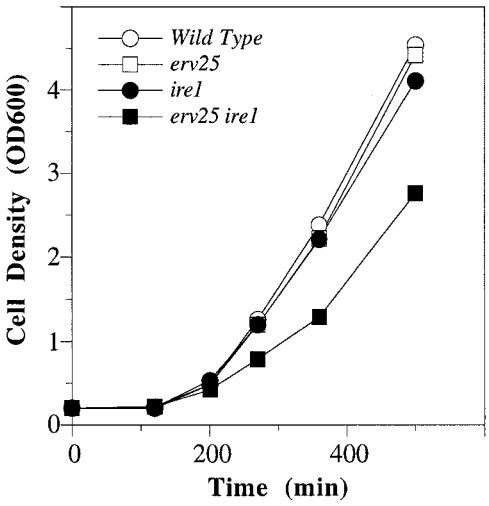

Figure 3. Growth rate is decreased in an *erv25*Δ *ire1* double mutant. The optical density at 600 nm was determined for FY834 (WT), CBY114 (*erv25*), CBY427 (*ire1*), and CBY425 (*ire1 erv25*) in rich media at 30^{O}C and plotted as a function of time.

functional p24 complex. Previous reports have shown that increasing functional *ERD2* expression can suppress trafficking mutants that secrete Kar2p (Semenza *et al.*, 1990). Therefore, we tested whether increased expression of Erd2p could suppress the Kar2p secretion phenotype of an *erv25*Δ strain, indicating whether Erd2p was functional. As seen in Figure 2, an *erv25*Δ strain harboring a 2 μ version of *ERD2* contained at least10-fold less Kar2p in the extracellular medium than an *erv25*Δ strain after 3 h of growth. As a positive control, the *sec22-3* strain shown previously to secrete Kar2p, was suppressed approximately sixfold by overexpression of Erd2p at the 3-h time point. The data indicate *ERD2* overexpression was able to increase the capacity of the HDEL retrieval system in *erv25*Δ and *sec22-3* strains. These results suggested that the p24 complex was unlikely to play a role in promoting the association of HDEL proteins to Erd2p. Together with the trafficking studies, it appeared that Erd2p cycles properly and was capable of retrieving HDEL proteins in a p24 deletion strain but that the Erd2p-dependent retrieval pathway was saturated and could not prevent secretion of Kar2p.

Deletion of ERV25 and IRE1 Reduces Growth Rate

ERD2 is an essential gene suggesting that retrieval of HDEL proteins is vital. However, removal of the HDEL sequence from Kar2p (*KAR2*Δ*HDEL*) does not result in loss of cell viability. Because *KAR2* is also an essential gene, cell viability appears to be maintained by increasing Kar2p synthesis (Semenza *et al.*,. 1990). This increased Kar2p synthesis depends on activation of the UPR because *KAR2*Δ*HDEL* displays a synthetic lethal relationship with *ire1*Δ, an ER localized transmembrane kinase that activates the UPR (Beh and Rose, 1995). In other words, a *KAR2*Δ*HDEL* strain survives because the cell compensates for loss of Kar2p through activation of the UPR and increased *KAR2* expression. Based on these published findings, we sought to determine whether a p24 deletion strain would rely on *IRE1* for growth because Kar2p was secreted from these strains. To test this possibility, we backcrossed *erv25*Δ*::HIS3* and *ire1*Δ*::URA3* strains and dissected individual asci. In all cases, dissection of asci resulted in four viable spores when grown on rich media at temperatures ranging from 25 to 37°C. Further quantitative analyses were performed by measuring the growth rates of an isogenic set of spores (WT, *erv25*Δ, *ire1*Δ, and *erv25*Δ *ire1*Δ) at 30°C. As seen in Figure 3, growth of the individual *erv25*Δ or *ire1*Δ strains did not differ from that of the wild-type, however, there was a detectable reduction in the growth rate of the *erv25*Δ *ire1*Δ double mutant strain. Based on exponential fitting of these data, the doubling time of the wild-type strain was calculated to be 92 min ($R = 0.99$) compared with 124 min ($R = 0.99$) for the double mutant. An almost identical decrease in growth rate was observed with an *emp24*Δ *ire1*Δ double mutant (our unpublished data), which displayed a growth rate of 123 min ($R = 0.99$). These results suggested that activation of the UPR was required for optimal growth although cell viability of p24 deletion strains did not depend on *IRE1*.

Loss of p24 Complex Activates the UPR

The properties on an *erv25*Δ *ire1*Δ double mutant strain suggested the UPR pathway was required for optimal growth in p24 deletion strains. To directly test whether the UPR was activated, we measured induction of the UPR from a reporter construct (pJC31) that contains the 22-bp UPRE from *KAR2* fused to *LacZ* (Mori *et al.*, 1992; Cox *et al.*, 1993). The fold activation from the UPRE can be determined in strains harboring this reporter construct through assay of β-galactosidase activity. As seen in Figure 4A, wild-type cells exposed for 60 min to β-mercaptoethanol (15 mM), activated transcription from the UPRE, resulting in an increase in β-galactosidase activity (Cox and Walter, 1996). Under this condition, cell growth continued at near normal rates. Similarly, an *erv25*Δ strain displayed a >2.4-fold increase in the amount of β-galactosidase compared with an

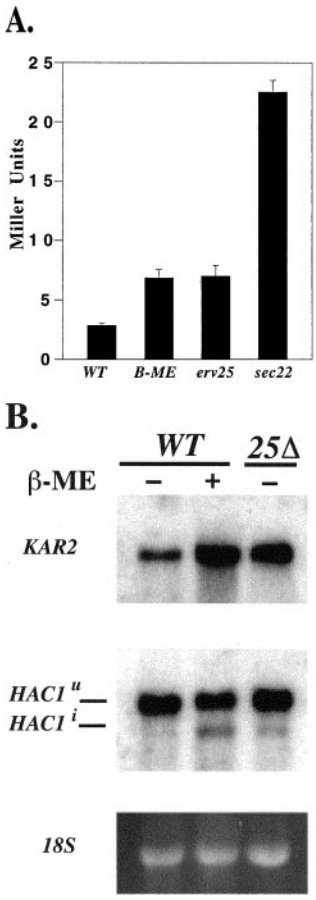

Figure 4. (A) Deletion of *ERV25* causes activation of the UPR. β-Galctosidase assays were performed on CBY635 (WT), CBY635 treated with 15 mM β-mercaptoethanol (β-ME) for 60 min, CBY637 (*erv25*Δ), and CBY748 (*sec22-3*). Activity is given in Miller Units and represents the average of seven independent determinations with SE. There is a significant difference ($p < 0.0001$) between the wild-type and *erv25*Δ strain. (B) Northern blot analysis of a FY834 (WT) and CBY114 (25Δ) with (+) or without (−) 15 mM β-mercaptoethanol (β-ME) treatment. Blots were probed for *KAR2* and *HAC1* messages with ^{32}P-labeled probes. Spliced (*HAC1*^{i}) and unspliced (*HAC1*^{U}) were observed. Ethidium stained 18 S rRNA serves as a loading control.

untreated wild-type strain ($p < 0.0001$), indicating activation of the UPRE. There was not a significant difference in activation between the *erv25*Δ strain with a wild-type strain exposured to β-mercaptoethanol for 60 min; however, longer treatments with this reducing agent resulted in significantly greater activation of the UPRE (our unpublished data). Furthermore, the amount of β-galactosidase activity measured in an *emp24*Δ *erv25*Δ strain was not significantly different from the *erv25*Δ strain alone (our unpublished data).

Interestingly, a strain with the *sec22-3* allele exhibited a strong activation of the UPR (>7-fold) as measured from this reporter construct. As reported previously (Semenza *et al.*, 1990) and shown in Figure 2, *sec22* strains secreted significant amounts of Kar2p into the medium and these cells apparently manage this situation through induction of the UPR. Sec22p may act in retrograde transport of proteins from the Golgi to the ER (Spang and Schekman, 1998); therefore, retrieval of HDEL proteins may be hindered in a *sec22-3* strain and cause increased Kar2p secretion. Alternatively, an ER accumulation of secretory proteins in *sec22-3* at permissive temperatures could lead to a proliferation of the ER and activation of the UPR. We favor this second interpretation because other *sec* mutants examined (e.g., *uso1-1*) exhibited varying degrees of UPR activation (our unpublished data). However, the amount of Kar2p secreted in an *erv25*Δ strain was slightly more than a *sec22-3* strain (Figure 2), whereas activation of the UPR was modest in *erv25*Δ and strong in *sec22-3.* These results suggested the manner in which *sec22* mutants and p24 deletion strains transport and/or dispose of up-regulated Kar2p expression may be distinct. These differences could be accounted for by a general secretory block in the *sec22* strain grown at this temperature or distinct compensatory changes in gene expression under control of the UPR (Travers *et al.*, 2000).

To provide further evidence for activation of the UPR in an *erv25*Δ strain, we monitored the levels of *KAR2* and spliced *HAC1* mRNA. Increases in these message levels are characteristic of an activated UPR (Sidrauski and Walter, 1997). As seen in Figure 4B, *KAR2* message was significantly elevated and spliced *HAC1* message was modestly elevated in an *erv25*Δ strain. A similar result was observed for wild-type cells treated with 15 mM β-mercaptoethanol. Based on these collective results, we conclude that loss of the p24 complex activates the UPR.

Activation of the UPR Increases Kar2p Secretion

The p24 deletion mutants and certain ER/Golgi *sec* mutants have a constitutively active UPR and secrete Kar2p. We next considered the possibility that activation of the UPR would lead to increased secretion of HDEL proteins. To test this idea, we activated the UPR by exposure to 15 mM β-mercaptoethanol and measured Kar2p secretion (Figure 5A). The level of extracellular Kar2p from treated cells was at least 10-fold greater than untreated cells after 3 h and was comparable to the amount secreted from an *erv25*Δ strain. In contrast, the intracellular levels of Kar2p were relatively constant in wild-type, β-mercaptoethanol treated and *erv25*Δ strains compared with the cytosolic marker protein Gdi1p (Figure 5B). Importantly, the extracellular Kar2p detected from cells treated with β-mercaptoethanol represents secreted material and was not due to cell lysis because intracellular markers were not increased in the culture medium. We have also observed that treatment with other activators of the UPR (e.g., 5 μg/ml tunicamycin) increased secretion of Kar2p into the culture medium (our unpublished data). Therefore, Kar2p secretion appeared to be a general phenotype of UPR activation. We chose 15 mM β-mercaptoethanol for additional studies (see below) because this relatively mild UPR activator had a modest effect on growth rate and secretion.

Based on these results, we speculated that UPR activation and subsequent up-regulation of HDEL proteins surpasses the capacity of both the ER retention and Erd2p-dependent retrieval processes. Previous studies have demonstrated that Erd2p-dependent retrieval is saturable when an HDEL-tagged version of a secretory protein (pro-α-factor) was

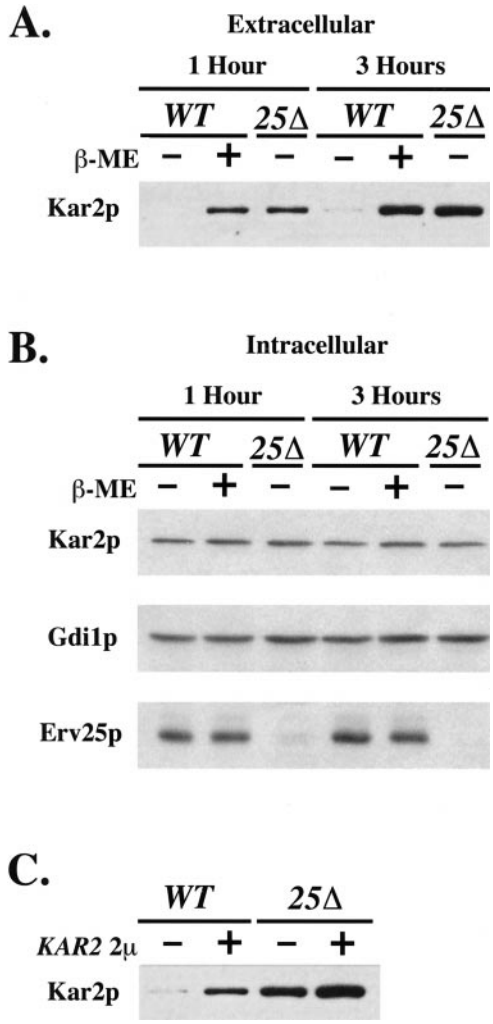

Figure 5. Activation of the UPR or overproduction of Kar2p increases extracellular Kar2p secretion. (A) Amount of secreted Kar2p from FY834 (WT) cells treated with (+) and without (−) 15 mM β-mercaptoethanol (β-ME) compared with CBY114 (25Δ). (B) Intracellular levels of Kar2p, Gdi1p, and Erv25p from equivalent amounts of cells (based on OD_{600}) grown as in A. (C) Extracellular Kar2p from FY834 (WT) and CBY114 (25Δ) that were transformed (+) or not (−) with a 2 μ *KAR2* plasmid. Proteins contained in the cell culture supernatant were concentrated by TCA precipitation and Kar2p was detected by immunoblot.

expressed (Townsley *et al.*, 1994). To determine whether overexpression of an endogenous HDEL protein was capable of saturating Erd2p retrieval, we transformed yeast strains with a 2 μ version of *KAR2* and measured levels of Kar2p secreted into the culture medium. In Figure 5C, wild-type strains containing the *KAR2* plasmid secreted at least 11-fold more Kar2p than untransformed strains. This result indicated that overproduced Kar2p failed to be retained in the ER and/or retrieved from post-ER compartments. Interestingly, we found that *KAR2* overexpression alone activated the UPR as evidenced by a 2.1-fold increase in β-galactosidase activity from the UPRE reporter construct when cotransformed with the 2 μ version of *KAR2* (strain CBY983). Presumably the UPR is activated under this condition because of a decrease in ER retention of other HDEL proteins (e.g., Pdi1p) that are replenished through UPR activation. Even higher levels of Kar2p were secreted from an *erv25*Δ strain containing the *KAR2* plasmid than from an *erv25*Δ strain or a wild-type strain with the *KAR2* plasmid (Figure 5C). The higher level of Kar2p secretion was approximately additive and was coincident with a greater activation of the UPR as revealed by a 3.7-fold increase in β-galactosidase activity when measured from the UPRE reporter construct in this strain (strain CBY984). These observations suggested the possibility that deletion of p24 genes causes Kar2p secretion as a consequence of UPR activation and up-regulation of HDEL proteins. Heightened activation of the UPR (addition of 2 μ *KAR2*) results in even greater levels of extracellular Kar2p when in p24 deletion strains.

Increased Kar2p in COPII Vesicles upon UPR Activation

If UPR activation somehow saturates the capacity of ER retention, we predicted that the amount of HDEL proteins exiting the ER would increase when the UPR is activated. To directly measure this level, we performed in vitro assays that reproduce COPII budding and cargo selection from ER membranes as in Figure 1. For these experiments, microsomes were prepared from a wild-type strain, an *erv25*Δ strain, or from a wild-type strain treated with 15 mM β-mercaptoethanol for 1 h. COPII-budded vesicles from these microsomes were isolated and the level of individual proteins packaged into vesicles monitored by immunoblot (Figure 6). The efficiency of incorporation for each protein was calculated as a percentage of the total by densitometry. From wild-type microsomes, we detected minor amounts of Kar2p (0.4% of total) or Sec61p in ER-derived vesicles, whereas Bos1p (10%), Erv25p (9.3%), and gp-α-factor (16%) were efficiently packaged (Figure 6A). In contrast, microsomes prepared from an *erv25*Δ strain budded COPII vesicles that contained sixfold more Kar2p (2.4%) but similar levels of Bos1p (11%) and gp-α-factor (13%). For the analysis of Kar2p and gp-α-factor by this method, vesicle preparations were treated with trypsin to ensure detection of protease-protected lumenal species. Clearly, the percentage of exported Kar2p increased when vesicles were budded from p24 deletion membranes, but also the ratio of Kar2p to Bos1p was greater in the deletion strain (0.22) compared with the wild-type strain (0.04). A similar result was obtained if Kar2p was compared with other vesicle proteins (i.e., gp-α-factor). These results demonstrated that more Kar2p was incorporated per COPII vesicle marker when the membrane source was from an *erv25*Δ strain.

We performed a similar analysis on microsomes prepared from wild-type cells that had been treated with 15 mM β-mercaptoethanol for 1 h to activate the UPR. As seen in Figure 6B, microsomes remained competent for budding after this treatment and more Kar2p was contained in COPII vesicles prepared from treated than untreated cells. Again, the ratio of Kar2p to gp-α-factor or Bos1p indicated an increase in the level of Kar2p per vesicle. Notably, Erv25p was present and efficiently packaged into vesicles after β-mercaptoethanol treatment yet Kar2p was not excluded from these vesicles. Therefore, p24 proteins do not appear to

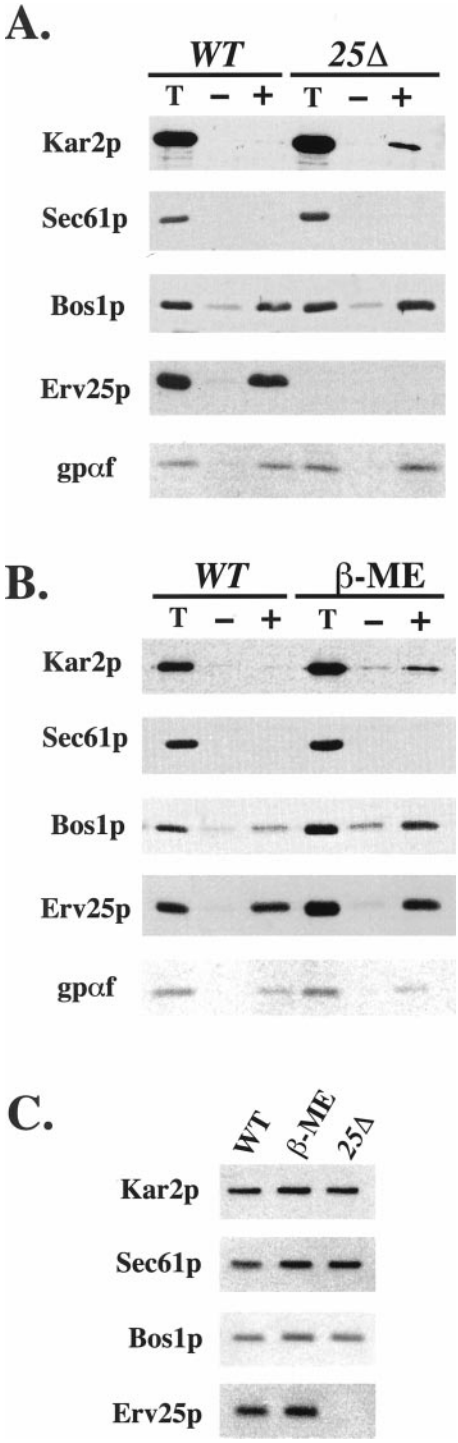

Figure 6. Deletion of *ERV25* or activation of the UPR increases the amount of Kar2p in COPII vesicles. (A) Reconstituted COPII budding reactions were performed on ER membranes isolated from FY834 (*WT*) and CBY114 (*25*Δ). Lanes labeled T represent one-tenth of the total membranes used in a budding reaction, minus (−) lanes indicate the amount of vesicles formed in the absence of the purified COPII components, and plus (+) lanes indicate vesicles produced when COPII proteins are added. Total membranes and budded vesicles were collected by centrifugation, resolved on polyacrylamide gels, and immunoblotted for indicated proteins. The Kar2p detected represents protease protected material (see MATERIALS AND METHODS) and [^{35}S]-glyco-pro-α-factor (gpαf) was monitored by fluorography. (B) As in A except ER membranes were prepared from FY834 (WT) or FY834 treated with 15 mM β-mercaptoethanol (β-ME), to activate the UPR. (C) Equal amounts of ER membranes (microsomes) blotted with indicated markers to compare levels of Kar2p.

prevent Kar2p from entering COPII vesicles when HDEL proteins are induced by activation of the UPR.

To determine whether the increase in Kar2p export from the ER correlated with an increase in ER levels of Kar2p, we directly compared microsomes prepared from wild-type, β-mercaptoethanol-treated, and *erv25*Δ strains (Figure 6C). Microsomal levels of Kar2p were monitored by immunoblot with Sec61p and Bos1p as loading controls. We did not detect significant Kar2p increases in microsomes prepared from an *erv25*Δ strain or a wild-type strain treated with β-mercaptoethanol. This result is consistent with our previous analysis of whole cells (Figure 5B). However, we probably would not be able to detect small changes in Kar2p levels by this method. One interpretation of these results is that Kar2p and other HDEL proteins are normally expressed at a threshold level that allows for efficient ER retention. When this level is exceeded, excess Kar2p is exported from the ER, surpasses Erd2p-dependent retrieval, and is secreted from cells. We would not expect to detect a 2% increase in Kar2p above endogenous levels in the ER fraction but can readily detect a 2% increase in Kar2p in COPII vesicles.

Further Analysis of the erv25Δ/ire1Δ Double Mutant

Loss of p24 function could cause a small amount of HDEL proteins to leak from the ER leading to activation of the UPR, or an ER accumulation of secretory proteins due to p24 deletion could activate the UPR. Either sequence of these events would apparently result in saturation of retention and retrieval processes leading to Kar2p secretion. The next series of experiments attempts to distinguish between these possibilities. If the increased level of Kar2p detected in COPII vesicles from an *erv25*Δ strain was due to activation of the UPR, we reasoned that silencing the UPR by *IRE1* deletion should diminish Kar2p export. To experimentally test this idea, we first compared budding reactions from an *ire1*Δ and an *erv25*Δ *ire1*Δ double mutant (Figure 7). Microsomes from the *ire1*Δ strain were fully competent for COPII budding in vitro because [^{35}S]-gp-α-factor, Erv25p, and Bos1p were efficiently packaged into vesicles. However, repeated isolation of microsomes from the *erv25*Δ *ire1*Δ double mutant strain yielded membranes that were not fully active in COPII budding reactions but were functional for translocation and core glycosylation of [^{35}S]-prepro-α-factor. Budding from the double mutant as measured by the percentage of release of [^{35}S]-gp-α-factor was approximately one-third the level observed for fully active microsomes. Elevating the concentration of COPII proteins or performing the budding reaction with semi-intact cell membranes (Baker *et al.*, 1988) did not alleviate this defect (our unpublished results). Regardless, we did not observe COPII-dependent budding of Kar2p from *erv25*Δ *ire1*Δ microsomes and speculate that we

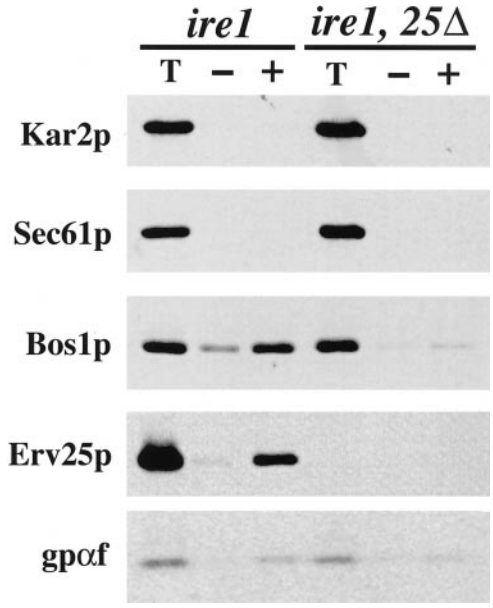

Figure 7. COPII budding is decreased in an *erv25*Δ *ire*Δ double mutant. (A) Reconstituted COPII budding reactions were performed as in Figure 6 except ER membranes were prepared form CBY427 (*ire1* Δ*) and CBY425 (*ire1 Δ, *25*Δ).

would be able to detect this amount if comparable to an *erv25*Δ strain that budded at one-third the wild-type level. From these results, we conclude that silencing *IRE1* in a p24 deletion strain somehow influences COPII budding from ER membranes. The experiment may indicate that Kar2p export from the ER is diminished in a p24 deletion strain when the UPR is silenced, however this interpretation is compromised because of the reduced budding efficiency.

If deletion of *IRE1* in the *erv25*Δ strain reduces the level of Kar2p export from the ER, we anticipated that less would be secreted into the extracellular medium. Indeed, immunoblot analysis of the culture medium from single and double mutant strains indicated combining the *ire1*Δ with *erv25*Δ blocked Kar2p secretion (Figure 8A) but did not alleviate ER accumulation of Gas1p (Figure 9). In the *erv25*Δ *ire1*Δ strain, some extracellular Kar2p could be detected above wild-type levels (Figure 8A). However, this may be true secretion or due to a small amount of cell lysis that occurred in the *erv25*Δ *ire1*Δ strain because minor amounts of cytosolic markers, such as Ssa1p (a heat shock protein 70 protein) and Gdi1p (a Rab-specific GDP dissociation inhibitor) were also detected in the culture fluid of this strain (Figure 8A). The general pattern observed by protein staining of extracellular proteins was similar in these four strains (Figure 8C) except that additional proteins and background staining appear in the *erv25*Δ *ire1*Δ strain, presumably due to cell lysis. The observed cell lysis of the double mutant may account for the decreased growth rate observed in Figure 3. However, the reduction in Kar2p secretion from the *erv25*Δ *ire1*Δ strain appears greater than the 1.3-fold reduction in growth rate exhibited by this strain. Furthermore, comparable amounts of the other major extracellular secretory proteins were detected in all four strains, indicating secretion was near normal in the double mutant. When we monitored intracellular

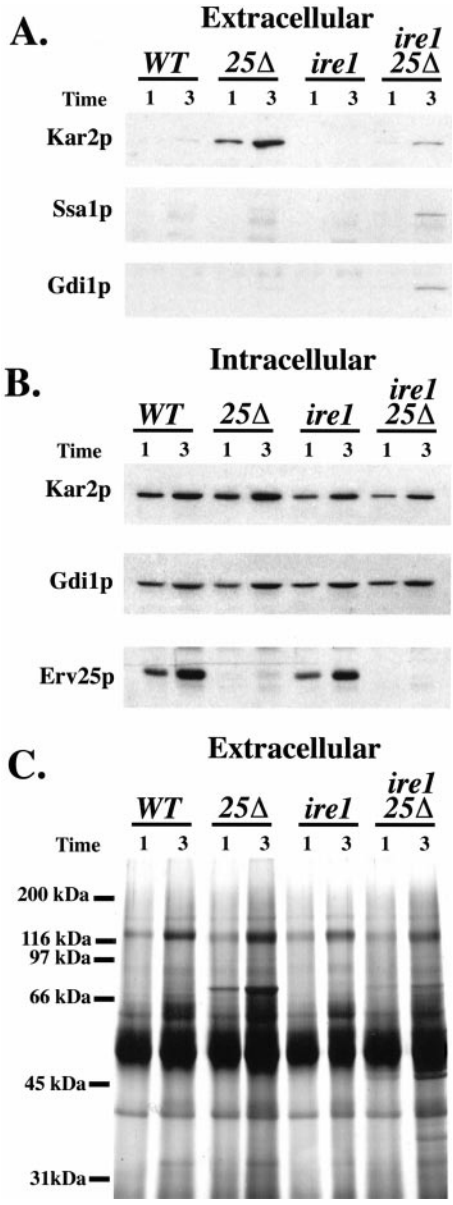

Figure 8. Deletion of *IRE1* prevents Kar2p secretion from an *erv25*Δ strain. (A) Amount of extracellular Kar2p in cultures from FY834 (*WT*), CBY114 (*erv25* Δ*), CBY427 (*ire1 Δ), and CBY425 (*erv25* Δ ire1 Δ) after 1 and 3 h. Proteins contained in the cell culture supernatant were concentrated by TCA precipitation and Kar2p was detected by immunoblot. (B) Intracellular levels of Kar2p, Gdi1p, and Erv25p from equal volumes of cell cultures grown as in A. (C) The same samples of precipitated proteins as in A were resolved on 12.5% SDS-PAGE and silver stained.

levels of Kar2p at these time points, we did not detect any significant increases however modest decreases in Kar2p were observed in *ire1*Δ strains (Figure 8B). These observations suggest that most of the Kar2p secreted by an *erv25*Δ strain depends on *IRE1*.

Finally, we measured the amount of Kar2p secreted by wild-type and *ire1*Δ strains that were treated with 15 mM

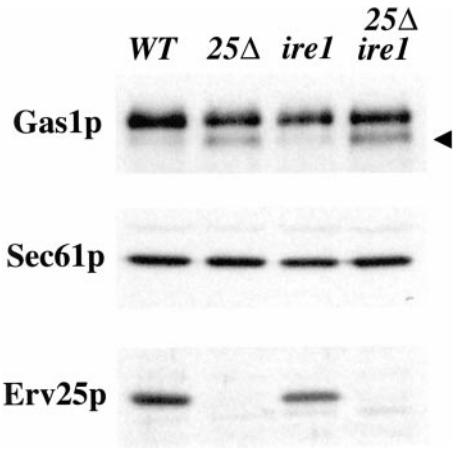

Figure 9. Immunoblot analysis of wild-type and deletion strains. Whole cell membranes prepared from FY834 (*WT*), CBY114 (*erv25* Δ), *CBY427 (*ire1 Δ), and CBY425 (*erv25* Δ ire1 Δ) strains after 3 h. Membrane proteins were resolved on polyacrylamide gels and immunoblotted for Gas1p, Sec61p (loading control), and Erv25p. The arrowhead indicates the ER form of Gas1p.

β-mercaptoethanol to activate the UPR pathway. We reasoned that if general induction of the UPR causes Kar2p secretion, silencing this response by *IRE1* deletion should also block the appearance of extracellular Kar2p. As seen in Figure 10A, *IRE1* deletion blocked Kar2p secretion by cells treated with β-mercaptoethanol. This treatment did not cause cell lysis or stop secretion over the 3-h time course that we monitored because comparable amounts of most extracellular secretory proteins were detected in the culture supernatant (Figure 10B). Treatment with this reductant did decrease the level of some secretory proteins (note asterisk at 120 kDa) and may be due to impaired disulfide bond formation of this secretory protein in the ER. In summary, extracellular secretion of Kar2p caused by p24 deletion or treatment with reducing agents depended largely on *IRE1*.

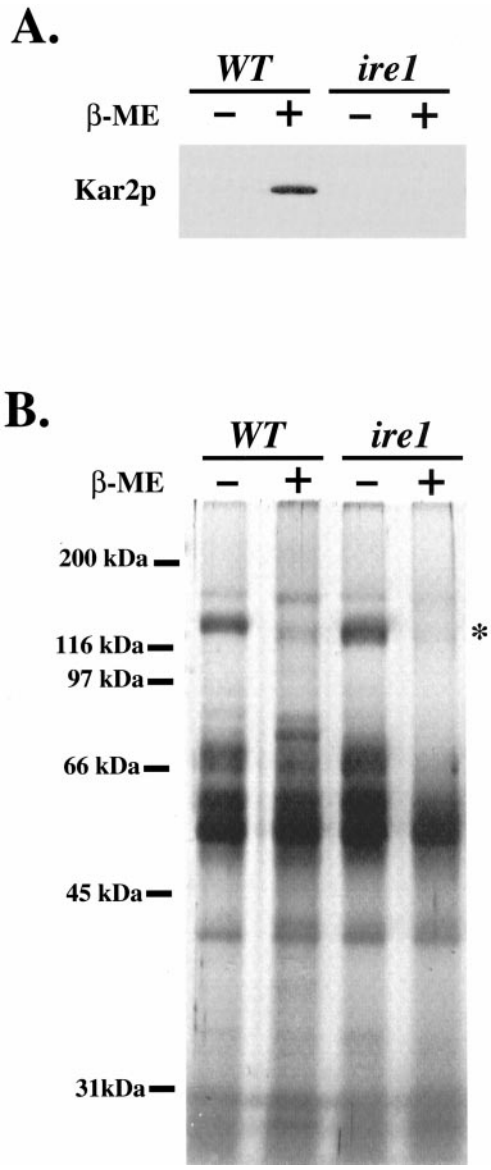

Figure 10. Deletion of *IRE1* prevents Kar2p secretion from cells treated with β-mercaptoethanol. (A) Amount of extracellular Kar2p from FY834 (*WT*) or CBY427 (*ire1* Δ) cells treated with (+) and without (−) 15 mM β-mercaptoethanol (β-ME) for 3 h. Proteins contained in the cell culture supernatant were concentrated by TCA precipitation and Kar2p was detected by immunoblot. (B) The same samples of precipitated proteins as in A were resolved on 12.5% SDS-PAGE and silver stained.

DISCUSSION

In this report, we investigated the pathways involved in extracellular secretion of Kar2p when cells lack a functional p24 complex. In *erv25*Δ strains, the Golgi-localized HDEL receptor Erd2p trafficked properly and was competent for returning HDEL proteins that had escaped the ER. In the absence of p24 function, however, the Erd2p-dependent retrieval pathway appeared saturated and failed to retrieve Kar2p, a condition that was partially alleviated by increasing the expression level of Erd2p. Reconstituted budding assays in an *erv25*Δ strain suggested that this saturation was due to an increase in the amount of Kar2p, and presumably other HDEL proteins, exported from the ER in COPII vesicles. We found that cells lacking a functional p24 complex exhibited an activated UPR. In addition, extracellular secretion of Kar2p in p24 deletion strains depended in large part on activation of the UPR because silencing this pathway through *IRE1* deletion greatly diminished Kar2p secretion. Finally, we report that other known activators of the UPR caused extracellular secretion of Kar2p and that Kar2p secretion is probably a hallmark of UPR activation.

Our experiments were performed in yeast cells that lack Erv25p and/or Emp24p and we find that these single or double mutants produced identical results. In the course of these studies, yeast strains that lack four (Marzioch *et al.*, 1999) or all eight (Springer *et al.*, 2000) of the yeast p24 family members were characterized and displayed secretion phenotypes that are indistinguishable from the single *ERV25* or

ERV24 deletions. Deletion of *ERV25* or *EMP24* destabilizes additional members of the heteromeric p24 complex and apparently leads to a complete loss of p24 function (Belden and Barlowe, 1996; Marzioch *et al.*, 1999; Springer *et al.*, 2000). We do not believe that destabilized p24 protein subunits in single deletion strains cause activation of the UPR because deletion of *ERV25* or all eight p24 genes produces identical phenotypes with respect to extracellular secretion of Kar2p (Springer *et al.*, 2000) and presumably UPR activation. Therefore, we speculate that an accumulation of secretory cargo in the ER activates the UPR although we cannot exclude the possibility that there are other non-p24 components of the hetromeric p24 complex that accumulate as unfolded species in the octuple mutant and influence the UPR pathway.

Two recent reports suggested that disruption of p24 function did not induce the UPR pathway in yeast (Springer *et al.*, 2000) or mammalian cells (Rojo *et al.*, 2000). We provide five experimental results that support a connection between the UPR pathway and p24 function in yeast. First, we find that the growth rate of p24 deletion strains was significantly reduced when *IRE1* was deleted (Figure 3). Second, in an *erv25*Δ strain we detected a greater than 2-fold activation of the UPR when β-galactosidase activity was measured from a reporter construct that places *lacZ* under the control of the UPRE (Figure 4). Third, we document that *KAR2* and spliced *HAC1* message levels are elevated in an *erv25*Δ strain (Figure 4B). Fourth, we find that microsomal membranes prepared from an *erv25*Δ *ire1*Δ double mutant strain were specifically compromised for vesicle budding in vitro, however, neither of these single mutations caused a defect (Figure 7). Fifth, silencing the UPR by *IRE1* deletion blocked Kar2p secretion from p24 deletion strains (Figure 8). In accord with previously reported findings (Springer *et al.*, 2000), we were unable to detect UPR activation in our p24 deletion strains from the *lacZ* reporter on X-Gal plates and we did not detect a temperature-sensitive phenotype when combining the p24 deletions with *ire1*Δ. However, when we measured β-galactosidase activity from cell lysates or followed the logarithmic phase growth rates of specific strains, reproducible and statistically significant effects were observed. Furthermore, we detected striking differences in the amount of Kar2p secreted by an *erv25*Δ strain compared with an *erv25*Δ *ire1*Δ strain and the in vitro budding efficiency of this double mutant was clearly distinct from either of the single mutants. Therefore, we believe these discrepancies can be accounted for by the assays used to detect UPR induction or that different strain backgrounds will influence these observations.

We observed that a general feature of UPR activation was extracellular secretion of Kar2p and probably other soluble HDEL-containing ER proteins that are induced by this stress response pathway. Treatment with chemical agents that interfere with protein folding in the ER (β-mercaptoethanol or tunicamycin) led to induction of the UPR and extracellular secretion of Kar2p. Overexpression of *KAR2* from a 2 μ plasmid also induced the UPR and caused Kar2p secretion. Based on these observations, we speculate that soluble ER residents are normally maintained at a threshold level and when elevated, ER retention and Erd2p-dependent retrieval mechanisms are surpassed. We are limited in our understanding of ER retention mechanisms but as previously suggested, *IRE1* could coordinate ER protein with ER membrane biosynthesis under normal conditions to achieve efficient retention (Cox *et al.*, 1997). Other conditions resulting in Kar2p secretion may represent an activation of the UPR. In keeping with this idea, we found that the *sec22-3* and *uso1-1* mutants induced the UPR and secreted extracellular Kar2p when grown at semipermissive temperatures. Other *sec* mutants that secrete Kar2p (Semenza *et al.*, 1990) probably possess an activated UPR. Further work will be needed to distinguish whether secretion of ER-resident proteins is beneficial in coping with accumulated secretory cargo or is simply a consequence of UPR activation. In considering this question, it seems notable that *IRE1* was required for optimal growth of p24 deletion strains, suggesting the UPR helps these cells manage a loss of p24 function. UPR induction in these strains could facilitate disposal of accumulated cargo through ER-associated protein degradation (McCracken and Brodsky, 1996) and/or accelerate transport from the ER for normal secretion or for degradation in the vacuole.

Do our findings provide insight on the function of p24 proteins? Several models have been offered on their role in the early secretory pathway (reviewed by Kaiser, 2000). First, the lumenal domains of these transmembrane proteins have been proposed to act as cargo receptors that bind to secretory cargo and link lumenal cargo to vesicle coat complexes (Schimmoller *et al.*, 1995; Muñiz *et al.*, 2000). Second, p24 proteins could act as negative regulators of vesicle budding, delaying the budding process to allow for more efficient segregation of cargo away from ER residents (Elrod-Erickson and Kaiser, 1996). Third, these molecules have been proposed to act as structural components of vesicles (Bremser *et al.*, 1999), of ER (Lavoie *et al.*, 1999), or of Golgi membranes (Rojo *et al.*, 2000) that could create specialized packaging zones. Fourth, the p24 proteins could act as steric exclusion devices occupying space within the lumen of vesicles, thereby blocking entry of soluble ER residents (Springer *et al.*, 2000; Kaiser, 2000). In considering the first and third models, our data seem consistent with the possibility that deletion of p24 proteins initially cause an accumulation of secretory cargo in the ER. Accumulated cargo may then elicit the UPR and increase the expression levels of ER chaperones, resulting in saturation of ER retention and post-ER retrieval processes. Our data may also be interpreted in a manner that is consistent with the second and fourth models, whereby p24 proteins act initially in retention of ER resident proteins. For example, Kar2p could initially leak from the ER in p24 deletion strains and trigger the UPR. An activated UPR could again lead to saturation of ER retention and retrieval processes. Regardless of initial cause, UPR activation appears to be critical for extracellular secretion of Kar2p in p24 deletion strains because of the observed influence of IRE1 deletion. In considering these models, it may be important to note that Kar2p secretion appeared to depend on *IRE1* but was not strictly dependent on the presence or absence of p24 proteins. For example, treatment of wild-type strains with β-mercaptoethanol resulted in extracellular secretion of Kar2p and this level of secretion depended on *IRE1*. However, deletion of p24 proteins appeared to be doing something more than simply activating the UPR because ER forms of secretory cargo such as Gas1p

accumulated in p24 deletion strains and this effect was not reversed by *IRE1* deletion.

Clearly, further experimentation will be necessary to determine the function of p24 proteins in ER sorting. Support for the cargo receptor model has been provided by evidence demonstrating a direct association between Gas1p and the Emp24p-Erv25p complex (Muñiz *et al.*, 2000). To further test this model, it will be informative to determine whether the secretory cargo invertase, which also accumulates in p24 deletion strains, possesses an affinity for the p24 complex. Alternatively, p24 proteins could be essential for setting up specialized membrane zones such as transitional ER (Lavoie *et al.*, 1999) or lipid rafts (Bagnat *et al.*, 2000) where cargo concentration might occur. Experimental methods to test these ideas are also available. For example, formation of transitional ER sites can be monitored in certain model organisms (Rossanese *et al.*, 1999) after deletion of p24 genes. These and other approaches should lead us to an understanding of p24 function in the early secretory pathway.

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