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Role of the Unfolded Protein Response Pathway in Secretory Stress and Regulation of *INO1* Expression in *Saccharomyces cerevisiae*

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ABSTRACT

The unfolded protein response pathway (UPR) enables the cell to cope with the buildup of unfolded proteins in the endoplasmic reticulum (ER). UPR loss-of-function mutants, *hac1*Δ and *ire1*Δ, are also inositol auxotrophs, a phenotype associated with defects in expression of *INO1*, the most highly regulated of a set of genes encoding enzymes of phospholipid metabolism. We now demonstrate that the UPR plays a functional role in membrane trafficking under conditions of secretory stress in yeast. Mutations conferring a wide range of membrane trafficking defects exhibited negative genetic interaction when combined with *ire1*Δ and *hac1*Δ. At semipermissive temperatures, carboxypeptidase Y transit time to the vacuole was slower in Sec^- cells containing an *ire1*Δ or *hac1*Δ mutation than in Sec^- cells with an intact UPR. The UPR was induced in Sec^- cells defective in subcellular membrane trafficking events ranging from ER vesicle trafficking to distal secretion and in *erg6*Δ cells challenged with brefeldin A. However, the high levels of UPR induction observed under these conditions were not correlated with elevated *INO1* expression. Indeed, many of the Sec^- mutants that had elevated UPR expression at semipermissive growth temperatures failed to achieve wild-type levels of *INO1* expression under these same conditions.

THE unfolded protein response pathway (UPR) is a stress response pathway that is activated when unfolded proteins accumulate in the endoplasmic reticulum (ER; Cox *et al.* 1993; Cox and Walter 1996; Mori *et al.* 1992, 1993). In yeast, the UPR consists of three components: Ire1p, Hac1p, and Rlg1p. Ire1p is a unique ER transmembrane spanning protein kinase/endoribonuclease. Hac1p is a transcription factor that is required for expression of UPR-responsive genes, including protein-folding chaperones, such as Kar2p (BiP; Kohno *et al.* 1993; Mori *et al.* 1992, 1993; Nikawa and Yamashita 1992). When improperly folded proteins accumulate in the ER, Ire1p autophosphorylates, thereby activating the Ire1p endoribonuclease activity, which catalyzes the splicing of the *HAC1* mRNA (Cox and Walter 1996; Mori *et al.* 2000), followed by ligation by Rlg1p, a tRNA ligase (Sidrauski *et al.* 1996). Since only the spliced form of *HAC1* mRNA is effectively translated (Chapman and Walter 1997; Kawahara *et al.* 1997), this regulated splicing leads to expression of Hac1p and subsequent activation of transcription of genes such as *KAR2*, containing the unfolded protein-responsive element (UPRE) in their promoters.

Cells carrying *ire1*Δ, *hac1*Δ, or *rlg1-100* mutations are sensitive to drugs such as tunicamycin, which causes accumulation of misfolded proteins in the ER (Nikawa *et al.* 1996; Shamu and Walter 1996; Sidrauski *et al.* 1996; Cox *et al.* 1997). In addition, *ire1*Δ, *hac1*Δ, or *rlg1-100* mutants are inositol auxotrophs (Nikawa and Yamashita 1992; Cox *et al.* 1993; Nikawa *et al.* 1996; Sidrauski *et al.* 1996), a phenotype associated with defects in expression of genes related to phospholipid metabolism, especially *INO1*, the structural gene encoding *myo*-inositol 3-phosphate synthase (for review see Henry and Patton-Vogt 1998). *INO1* and coregulated genes of phospholipid metabolism contain the inositol-sensitive upstream activating sequence (UAS_{INO}) repeated element in their promoters and exhibit complex transcriptional regulation in response to a variety of environmental factors including the availability of soluble precursors of phospholipid metabolism such as inositol (Carman and Henry 1999). Wild-type yeast cells express *INO1* and other UAS_{INO}-containing genes at a high level when inositol is limiting in the growth medium and repress these same genes when inositol is plentiful (Hirsch and Henry 1986; Greenberg and Lopes 1996; Carman and Henry 1999; Loewen *et al.* 2004). Cox *et al.* (1997) reported that the UPR is activated in the absence of inositol and suggested that the activation of the UPR might be directly involved in the mechanism by which *INO1* transcription is activated when inositol is limiting.

In an earlier study, we explored the relationship between UPR induction and *INO1* expression in response to signals generated due to altered phospholipid metab-

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olism in mutants defective in the phosphatidylinositol (PI)/phosphatidylcholine (PC) transporter encoded by the *SEC14* gene (Chang *et al.* 2002). While the *SEC14* gene product is essential for viability, the growth and secretory defects of *sec14* mutants can be suppressed by mutations in the cytidine 5′-diphosphate (CDP) choline pathway for PC biosynthesis, such as *cki1*Δ, *pct1*Δ, and *cpt1*Δ (Cleves *et al.* 1991). Double mutants, such as *sec14^{ts} cki1*Δ, when shifted to a semipermissive or restrictive temperature for *sec14^{ts}*, exhibit multiple abnormalities in phospholipid metabolism, including both elevated PC turnover via a phospholipase D catalyzed route and elevated expression of *INO1* (Patton-Vogt *et al.* 1997; Sreenivas *et al.* 1998). Under these same conditions *sec14^{ts} cki1*Δ cells also exhibit high levels of UPR activation (Chang *et al.* 2002). These observations are consistent with the hypothesis of Cox *et al.* (1997) that the UPR and inositol responses are linked and are branches of the same signaling pathway. However, when the UPR mutations *ire1*Δ and *hac1*Δ were crossed into the *sec14^{ts} cki1*Δ background, elevated *INO1* expression was still observed at the *sec14^{ts}* semipermissive temperature, indicating that UPR activation is not required or responsible for the elevated *INO1* expression observed in *sec14^{ts} cki1*Δ cells (Chang *et al.* 2002).

The effects we observed in *sec14^{ts} cki1*Δ cells led us to question whether defects in other steps in the secretory pathway might also activate the UPR and/or influence *INO1* expression and, thus, provide further insights into the role of the UPR in the secretory pathway and in *INO1* regulation. In this study, we have examined the role of the UPR in membrane trafficking and in *INO1* expression under conditions of secretory stress induced by lesions in a number of transport steps of the secretory pathway. We report that cells defective in a wide range in membrane trafficking steps exhibit UPR activation and that a functional UPR plays a role in the survival of cells with inefficient membrane trafficking due to a wide range of secretory defects. Thus, the UPR appears to be essential to growth under conditions in which secretory capacity is diminished. However, Sec$^-$ mutants grown under the conditions that result in UPR activation exhibit reduced, rather than elevated, *INO1* expression. Thus, the relative levels of UPR activation and *INO1* transcription are not correlated under conditions of secretory stress.

MATERIALS AND METHODS

Media and growth conditions: Yeast extract peptone media with dextrose (YEPD) and synthetic medium, with (I$^+$) or without (I$^-$) inositol, or containing tunicamycin (tm) with or without leucine or uracil were prepared as previously described (Chang *et al.* 2002). Media containing brefeldin A (BFA; Sigma, St. Louis) were prepared as described by Graham *et al.* (1993) and Vogel *et al.* (1993).

Yeast strains: The genotypes and sources of strains are listed in Table 1. Yeast strains listed in Table 1 are of the S288C and W303 genetic backgrounds. Strains were constructed by standard tetrad analysis (Sherman *et al.* 1978; Rose *et al.* 1990). Briefly, HCY104, HCY105, HCY106, HCY107, HCY126, and HCY362 were obtained as meiotic spore colonies from diploid deletion strains obtained from the Research Genetics (Huntsville, AL) strain collection. To obtain an *ire1*Δ strain isogenic with the genetic background of Sec$^-$ strains used in this study, the HCY405 strain was constructed using PCR-based gene disruption of *IRE1* in the CKY8 genetic background. A 1.5-kb deletion cassette containing the bacterial KanMX gene was amplified by PCR using pFA6-KanMX4 as a template and IRE1-S1 and IRE1-S2 as the primers (Wach *et al.* 1994). The KanMX4-containing PCR fragment was included at each end, 45 bp (85–129) and 43 bp (3039–3081), of the *IRE1* ORF, resulting in replacement of the *IRE1* gene with KanMX. The 1.5-kb deletion cassette was transformed into CKY8 using the lithium acetate (LiAc) method as described previously (Hill *et al.* 1991). Transformants were selected on YEPD containing 200 mg/liter geneticin (YEPD + G418; Calbiochem, La Jolla, CA). To verify the correct ORF replacement, the size of the insert (1.5 kb) was checked by PCR using a set of primers flanking the disrupted gene (IRE1-S3 and IRE1-S4) and genomic DNA isolated from each of the putative deletion mutants as a template. HCY423 was obtained by mating HCY405 and CKY46. To obtain a *hac1*Δ mutant in the same genetic background, a *hac1*Δ meiotic spore generated from the heterozygous diploid strain collection (Research Genetics) was backcrossed to CKY49 to generate HCY126.

Double mutants, Sec$^-$ *ire1*Δ or Sec$^-$ *hac1*Δ, were constructed by crossing the various *ire1*Δ or *hac1*Δ strains to the various Sec$^-$ strains. HCY280 and HCY283 were obtained by mating HCY423 and RSY263. HCY427 and HCY428 were obtained by mating HCY423 and RSY267. HCY425 and HCY422 were obtained by mating HCY405 and CKY46. HCY437 and HCY 435 were obtained by mating HCY423 and RSY269. HCY442 and HCY444 were obtained from the mating between HCY423 and RSY271. HCY455 and HCY457 were obtained from the cross of HCY423 and RCY279. HCY448 and HCY446 were obtained by mating HCY423 and RCY281. HCY470 and HCY473 were obtained by mating HCY405 and JBY318. HCY178, HCY 179, HCY180, and HCY181 were obtained from the cross of HCY126 and CKY49. HCY462, HCY463, HCY465, and HCY 464 were the products of a cross between HCY362 and HCY 401. HCY495 and HCY496 were obtained from a mating between HCY107 and RCY243. Two sets of four spores containing the tetratype genotypes derived from a single tetrad (HCY462–HCY465 and HCY466–HCY469) were generated by crossing HCY362 (*erg6*Δ) to HCY401 (*ire1*Δ) and HCY030 (*hac1*Δ), respectively.

Transformation with pHAC1 and pHAC1^i: Various Sec$^-$ strains were transformed with the *HAC1*- and *HAC1^i*-containing plasmids (pHAC1 and pHAC1^i, CEN/*LEU2* marker; gifts from Dr. Kazutoshi Mori, Kyoto University, Kyoto, Japan). The *sec13*Δ strain, CKY480 (see Table 1 for the full genotype), carrying the wild-type *SEC13* gene on a plasmid (pRS316; CEN; *URA3* marker; provided by Dr. Chris Kaiser) was transformed with pHAC1^i or an empty vector (YCp50; CEN; *LEU2* marker). Since the *SEC13* gene is essential for growth, the *SEC13* null mutant is obligated to carry and express the wild-type *SEC13* gene on a plasmid for its survival. The double transformants simultaneously carrying pSEC13 and an empty vector or pSEC13 and pHAC1^i were grown for 1 day on plates containing synthetic medium lacking uracil and leucine and then replica plated onto plates lacking leucine and containing 5-fluoro-orotic-acid (5-FOA) and incubated for an additional 3 days at room temperature. The expression of the *URA3* gene is toxic to cells exposed to 5-FOA, forcing the transformants to lose

TABLE 1

Strains used in this study

Strain	Genotype	Source/reference
BJ8928	*MAT***a** *pep12::LEU2 his3 leu2 lys2 trp1 ura3*	Elizabeth Jones
RSY263	*MAT*α *sec12*^{ts}*-4 leu2-3,112 ura3-52*	Randy Schekman
RSY267	*MAT*α *sec16*^{ts}*-2 his4-619 ura3-52*	Randy Schekman
RSY269	*MAT*α *sec17*^{ts}*-1 his4-619 ura3-52*	Randy Schekman
RSY271	*MAT*α *sec18*^{ts}*-1 his4-619 ura3-52*	Randy Schekman
RSY279	*MAT*α *sec22*^{ts}*-3 his4-619 ura3-52*	Randy Schekman
RSY281	*MAT*α *sec23*^{ts}*-1 his4-619 ura3-52*	Randy Schekman
CKY480	*MAT*α *sec13-Δ1 ade2-101 ade3-24 leu2-3, 112 ura3-52 pSEC13 (CEN4; URA3)*	Chris Kaiser
CKY46	*MAT***a** *sec13*^{ts}*-1 his4-619 ura3-52*	Chris Kaiser
CKY49	*MAT***a** *sec13*^{ts}*-4 leu2-3,112 ura3-52*	Chris Kaiser
CKY8	*MAT*α *leu2-3,112 ura3-52*	Chris Kaiser
JBY318	*MAT***a** *sec6*^{ts}*-4 his4 leu2 ura3 GAL+*	Jeff Brodsky
RCY927	*MAT*α *sec21*^{ts}*-1 leu2-3,112 ura3-52*	Ruth Collins
RCY243	*MAT*α *sec1*^{ts}*-1 leu2-3,112 ura3-52*	Ruth Collins
RCY248	*MAT*α *sec4*^{ts}*-8 leu2-3,112 ura3-52*	Ruth Collins
RCY274	*MAT*α *sec2*^{ts}*-59 ura3-52*	Ruth Collins
RCY260	*MAT***a** *sec15*^{ts}*-1 leu2-3,112 ura3-52*	Ruth Collins
SHY625	*MAT*α *sec14*^{ts}*-3 his3 leu2 lys2 trp1 ura3*	Patton-Vogt *et al.* (1997)
HCY030	*MAT*α *hac1::URA3 his3 lys2 leu2 trp1 ura3*	Chang *et al.* (2002)
HCY104	*MAT***a** *his3 leu2 ura3*	This study
HCY105	*MAT*α *ire1::*KanMX *his3 leu2 ura3*	This study
HCY106	*MAT*α *his3 leu2 lys2 ura3*	This study
HCY107	*MAT***a** *ire1::*KanMX *his3 leu2 lys2 ura3*	This study
HCY126	*MAT*α *hac1::*KanMX *his3 leu2 lys2 ura3*	This study
HCY178	*MAT*α *sec13*^{ts}*-4 hac1::*KanMX *leu2 ura3*	This study
HCY179	*MAT***a** *his3 leu2 ura3*	This study
HCY180	*MAT*α *sec13*^{ts}*-4 his3 leu2 lys2 ura3*	This study
HCY181	*MAT***a** *hac1::*KanMX *leu2 lys2 ura3*	This study
HCY280	*MAT*α *sec12*^{ts}*-4 his4-619 ura3*	This study
HCY283	*MAT*α *sec12*^{ts}*-4 ire1::*KanMX *his4-619 leu2-3,112 lys2 ura3*	This study
HCY362	*MAT***a** *erg6::*KanMX *his3 leu2 met15 ura3*	This study
HCY401	*MAT*α *ire1::TRP1 ade2 his3 leu2 trp1*	Chang *et al.* (2002)
HCY405	*MAT*α *ire1::*KanMX *leu2-3,112 ura3-52*	This study
HCY422	*MAT*α *sec13*^{ts}*-1 ire1::*KanMX *his4-619 ura3*	This study
HCY423	*MAT***a** *ire1::*KanMX *ura3-52*	This study
HCY424	*MAT***a** *leu2 ura3*	This study
HCY425	*MAT*α *sec13*^{ts}*-1 his4-619 leu2 ura3*	This study
HCY427	*MAT*α *sec16*^{ts}*-2 his4-619 ura3*	This study
HCY428	*MAT***a** *sec16*^{ts}*-2 ire1::*KanMX *his4-619 ura3*	This study
HCY435	*MAT*α *sec17*^{ts}*-1 ire1::*KanMX*his4-619 ura3*	This study
HCY437	*MAT*α *sec17*^{ts}*-1 ura3*	This study
HCY442	*MAT***a** *sec18*^{ts}*-1 ire1::*KanMX *his4 ura3*	This study
HCY444	*MAT*α *sec18*^{ts}*-1 ura3*	This study
HCY446	*MAT***a** *sec23*^{ts}*-1 ura3*	This study
HCY448	*MAT***a** *sec23*^{ts}*-1 ire1::*KanMX*his4 ura3*	This study
HCY455	*MAT***a** *sec22*^{ys}*-3 his4 ura3*	This study
HCY457	*MAT*α *sec22*^{ts}*-3 ire1::*KanMX*ura3*	This study
HCY462	*MAT***a** *his3 leu2 met15 ura3*	This study
HCY463	*MAT***a** *erg6::*KanMX *ade2 his3 leu2*	This study
HCY464	*MAT*α *erg6::*KanMX *ire1::TRP1 his3 leu2 met15 trp1*	This study
HCY465	*MAT*α *ire1::TRP1 ade2 his3 leu2 trp1 ura3*	This study
HCY466	*MAT***a** *erg6::*KanMX *his3 leu2 met15 ura3*	This study
HCY467	*MAT***a** *hac1::URA3 his3 leu2 lys2 met15 trp1 ura3*	This study
HCY468	*MAT*α *erg6::*KanMX *hac1::URA3 his3 leu2 lys2 ura3*	This study
HCY469	*MAT*α *his3 leu2 trp1 ura3*	This study
HCY470	*MAT***a** *sec6*^{ts}*-4 his4 leu2 ura3*	This study
HCY473	*MAT*α *sec6*^{ts}*-4 ire1::*KanMX*his4 leu2 ura3*	This study
HCY495	*MAT*α *sec1*^{ts}*-1 his3 leu2 ura3*	This study
HCY496	*MAT*α *sec1*^{ts}*-1 ire1::*KanMX *leu2 lys2 ura3*	This study

the pSEC13 plasmid, thus obligating them to depend on expression of pHAC1[i] for survival.

β-Galactosidase assays: To assay UPRE-*CYC-lacZ* expression, strains were transformed to uracil prototrophy with pJC104, containing UPRE-*CYC-lacZ*, provided by Dr. Peter Walter (Cox and Walter 1996). Transformants were precultured to midlogarithmic phase of growth at 25° in synthetic medium lacking uracil. Cells were collected by centrifugation, washed with sterile dH_2O, and diluted to $OD_{600} = 0.1$ in medium lacking uracil and shifted to the designated temperature to grow for 3 additional hours. Samples (1 ml) were taken and analyzed for β-galactosidase activity using the Yeast β-galactosidase assay kit (Pierce, Rockford, IL). For UPRE-*CYC-lacZ* expression assay in BFA-treated *erg6*Δ cells, HCY104 and HCY362 were transformed with pJC104 (UPRE-*CYC-lacZ*). Cells were prepared as described above except that their initial OD_{600} was 0.5. Indicated amounts of BFA were added to medium lacking uracil and cells were grown at 30°, and β-galactosidase activity was measured as described above.

To assess *INO1* expression, strains were transformed to uracil prototrophy with pJH359 (*INO1-CYC-lacZ*) and transformants were precultured at 25° to midlog phase of growth in synthetic medium with (I^+) or without (I^-) inositol. Cells were then shifted to the indicated temperature for 3 hr and β-galactosidase was measured.

Northern blot analysis: Cells were precultured overnight to midlogarithmic phase of growth at 25° in YEPD medium. Wild-type cells were shifted to YEPD medium at 30° in the presence or absence of 1 mm tunicamycin. Sec^- strains were shifted to YEPD medium at their semipermissive temperatures. Isolation of RNA was performed by hot phenol extraction. Northern analysis was performed by running 10-μg samples of RNA loaded onto 1% agarose, 6% formaldehyde, 1× MOPS gels and capillary transferred to Nylon Plus (QIAGEN, Valencia, CA) membrane. Prehybridization and hybridization conditions were as described in Hirsch and Henry (1986). Hybridization probes for *ACT1* (Marykwas and Fox 1989) and *HAC1* were prepared using a riboprobe *in vitro* transcription system. *ACT1* was linearized with *Bam*HI and riboprobe was synthesized with SP6 RNA polymerase according to manufacturer's instructions (Promega, Madison, WI). The 449-bp *Sal*I fragment from YCp-*HAC1* was subcloned into pGEM1 (Promega) through the *Sal*I site (pGEM-*HAC1*). pGEM-*HAC1* was linearized with *Hin*dIII. Riboprobe was synthesized with T7 RNA polymerase according to manufacturer's instructions.

Spheroplast pulse-chase labeling and immunoprecipitation: Spheroplast labeling and immunoprecipitation were carried out essentially as described by Webb *et al.* (1997). All strains were grown at the temperatures indicated in Figure 5 and in the text to midlogarithmic phase ($OD_{600} = 0.6$–1.0) at 24° in either Wickerman's sulfate-free medium containing 200 μm $MgSO_4$ and 0.2% yeast extract (wild type, *sec13*^{ts}*-1*, and *sec13*^{ts}*-1 ire1*Δ strains) or synthetic complete medium lacking leucine (*sec13*^{ts}*-4 hac1*Δ pHAC1[i] transformants). Five OD_{600} units of cells per time point were collected and converted to spheroplasts in Wickerham's medium containing 1 m sorbitol and 1 mg/ml bovine serum albumin by digestion with 30 units/OD_{600} lyticase (Sigma) at room temperature for 45 min. Spheroplasts from wild-type, *sec13*^{ts}*-1*, and *sec13*^{ts}*-1 ire1*Δ strains were preincubated for 15 min at 30°; pulse labeled with 50 μCi Trans-^{35}S labeling reagent (ICN Radiochemicals, Irvine, CA) in spheroplasting medium for 5 min at 30°; and chased by adding 5 mm methionine and 1 mm cysteine, 0.2% yeast extract, and 2% glucose and incubating for 0, 10, 20, or 30 min at 30°. Pulse-chase labeling of spheroplasts from *sec13*^{ts}*-4 hac1*Δ pHAC1[i] transformants was carried out as described above except incubations were performed at 35°. Protein extracts were prepared by trichloracetic acid precipitation of cell pellets followed by resuspension and boiling with glass beads in suspension buffer (50 mm Tris-HCl pH 7.5, 1 mm EDTA, 1% SDS). After a fivefold dilution with IP dilution buffer (60 mm Tris-HCl pH 7.5, 6 mm EDTA, 190 mm NaCl, 1.25% Triton X-100), samples were precleared by centrifugation to remove insoluble material. Carboxypeptidase Y (CPY) was immunoprecipitated from each sample by incubating with rabbit anti-CPY antibodies (Rockland, Gilbertsville, PA) and protein A-sepharose CL-4B (Sigma) in IP buffer (50 mm Tris-HCl pH 7.5, 5 mm EDTA, 150 mm NaCl, 1% Triton X-100, 0.2% SDS). Washed immunoprecipitates were eluted with sample buffer, separated on 8% SDS polyacrylamide gels, and visualized by autoradiography. A digital image of the gel was acquired by scanning the autoradiograph. To obtain the signal intensity of each species of CPY, the digital image was analyzed by Kodak digital science 1D image analysis software (Eastman Kodak, Rochester, NY). The relative total labeling of CPY was estimated by summing up the signal intensity of p1, p2, and mature (mCPY) on each autoradiograph. The proportion of each species of was then calculated by dividing the separate signal intensity of p1, p2, or m by the sum of the intensity of the three bands.

RESULTS

Secretory stress activates the UPR: Secretory mutants affecting various membrane transport steps (for review of secretory pathway mutant defects, see Kaiser *et al.* 1997) were chosen for analysis of UPR induction and *INO1* expression under conditions of secretory stress. The strains analyzed include several carrying temperature-sensitive mutations conferring defects in formation of COPII vesicles affecting exit from the ER (*sec12*^{ts}*-4*, *sec13*^{ts}*-1*, *sec13*^{ts}*-4*, *sec16*^{ts}*-2*, and *sec23*^{ts}*-1*), three with defects in vesicle docking and fusion processes affecting the early stages of the secretory pathway (*sec17*^{ts}*-1*, *sec18*^{ts}*-1*, and *sec22*^{ts}*-3*), and one with a defect in COPI coatomer (*sec21*^{ts}*-1*). Five mutations conferring temperature-sensitive defects in distal secretion to the plasma membrane (*sec6*^{ts}*-4*, *sec1*^{ts}*-1*, *sec15*^{ts}*-1*, *sec2*^{ts}*-59*, and *sec4*^{ts}*-8*) and one deletion mutation conferring a nonlethal defect in vacuolar targeting, *pep12*Δ (Becherer *et al.* 1996), were also analyzed).

Activation of the UPR in wild-type and mutant strains (Figure 1A) was assayed using an UPRE-*CYC-lacZ* reporter gene, as described in materials and methods. Sec^- strains were assayed only under conditions at which they could grow continuously. Therefore, β-galactosidase activity is reported for only a subset of strains at 33° (Figure 1A). Wild-type cells exhibited the expected low levels of UPRE expression at 25° and 30°, and only a slight increase in UPRE expression was observed at 33° (Figure 1A). In contrast, virtually all of the temperature-sensitive Sec^- mutants showed elevated UPRE activation compared to wild type when grown at their semipermissive temperatures of 30° or 33° (Figure 1A).

Several of the Sec^- mutants showed elevated UPRE expression even at 25°. For example, among the strains defective in COPII vesicle formation, UPRE-*CYC-lacZ* activity was elevated >11-fold in the *sec16*^{ts}*-2* strain and

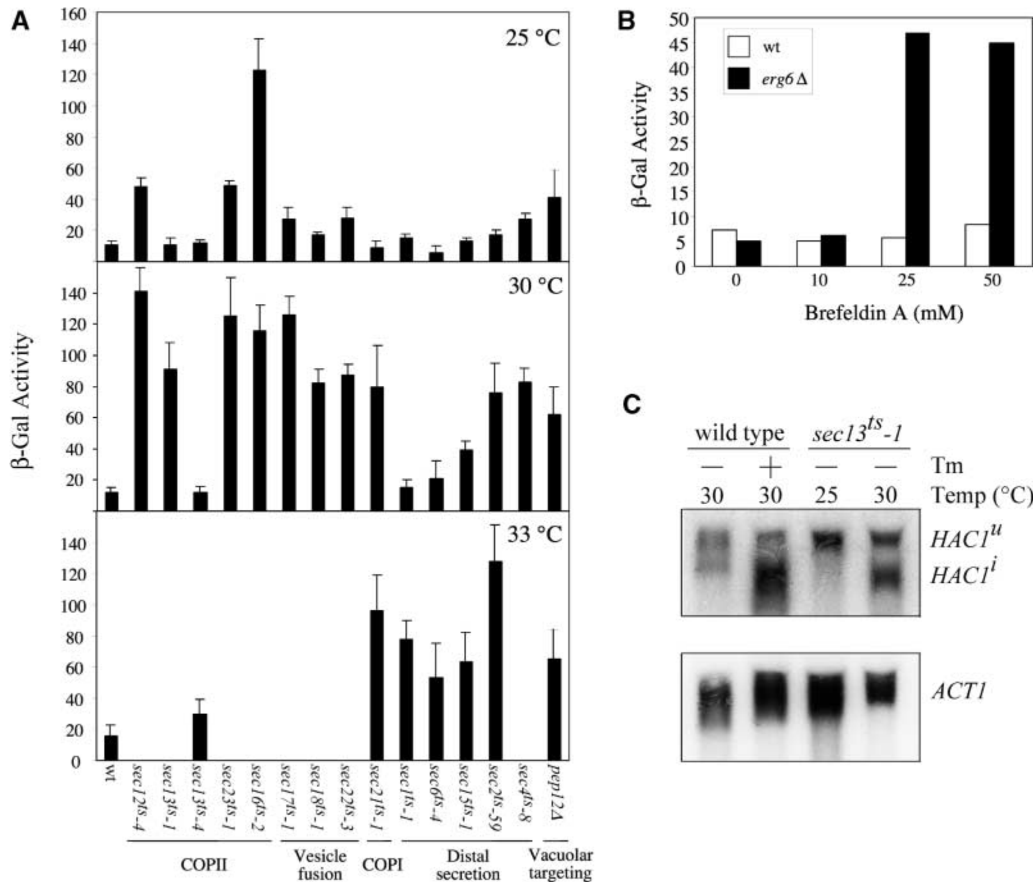

FIGURE 1.—Activation of the UPR in cells blocked in secretion and vacuolar targeting. (A) Transcription of UPRE is induced in mutants defective in membrane trafficking and transport. CKY8 (wt), RSY263 (*sec12*^{ts}*-4*), CKY 46 (*sec13*^{ts}*-1*), CKY49 (*sec13*^{ts}*-4*), RSY281 (*sec23*^{ts}*-1*), RSY 267 (*sec16*^{ts}*-2*), RSY269 (*sec17*^{ts}*-1*), RSY271 (*sec18*^{ts}*-1*), RSY279 (*sec22*^{ts}*-3*), RCY927 (*sec21*^{ts}*-1*), RCY243 (*sec1*^{ts}*-1*), RCY248 (*sec4*^{ts}*-8*), JBY318 (*sec6*^{ts}*-4*), RCY260 (*sec15*^{ts}*-1*), and BJY8928 (*pep12*Δ) were transformed with pJC 104 (UPRE-*CYC-lacZ*). Transformants were precultured at 20° to midlogarithmic phase of growth in medium lacking uracil and shifted to indicated temperatures. Samples were taken after 3 hr following the shift and analyzed for β-galactosidase activity as described in MATERIALS AND METHODS. β-Galactosidase activity unit was defined as OD_{420}/min/ml. (B) Transcription of UPRE is induced at 30° in BFA-treated *erg6*Δ cells. HCY 104 (wt) and HCY362 (*erg6*Δ) carrying pJC104 (UPRE-*CYC-lacZ*) were grown in uracil dropout medium containing 0, 10, 25, and 50 mM BFA. Samples were taken at 2 hr. β-Galactosidase activity unit was defined as OD_{420}/min/ml. (C) Splicing of *HAC1* transcripts is elevated in *sec13*^{ts}*-1* cells. Northern blot analysis was performed to monitor splicing of *HAC1* mRNA in *sec13*^{ts}*-1* cells (CKY49) grown at 25° and 30°. As a control, splicing of *HAC1* mRNA was also monitored in wild-type cells (CKY8) treated with 1 mM tunicamycin (Tm). Cells were precultured overnight in YEPD medium at 25° and shifted to indicated temperatures (for wild-type positive control, 1 mM Tm was added). Samples were taken after 3 hr following the temperature shift. *ACT1* mRNA is shown as a loading control.

>3-fold in the *sec12*^{ts}*-4* and *sec23*^{ts}*-1* strains, in comparison to wild type, at the permissive temperature of 25° (Figure 1A). At the semipermissive temperature of 30°, UPRE expression 7- to 10-fold higher than that of wild type was observed in *sec12*^{ts}*-4*, *sec13*^{ts}*-1*, *sec23*^{ts}*-1*, and *sec16*^{ts}*-2* mutants (Figure 1A). Strains carrying *sec13*^{ts}*-4* are capable of growing at temperatures up to 36°, and under these conditions, UPRE expression was elevated >7-fold compared to expression in the same strain at 25° (data not shown).

The *sec17*^{ts}*-1*, *sec18*^{ts}*-1*, and *sec22*^{ts}*-3* strains, which are defective in vesicle docking and fusion, also exhibited a five- to eightfold elevation in UPRE-driven β-galactosidase activity at 30° (Figure 1A). In the *sec21*^{ts}*-1* strain, which has a defect in COPI coatomer, UPRE expression was elevated more than fivefold at 30° and 33°, as compared to wild type (Figure 1A). UPRE expression three- to sevenfold higher than that of wild type was also observed at the semipermissive temperature of 30° or 33° in *sec15*^{ts}*-1*, *sec2*^{ts}*-59*, *sec1*^{ts}*-1*, *sec6*^{ts}*-4*, and *sec4*^{ts}*-8*, which are defective in distal secretion. The *pep12*Δ strain, which is defective in vacuolar targeting and is not temperature sensitive, exhibited UPRE expression levels approximately fourfold higher than wild-type grown under identical conditions at all temperatures tested (Figure 1A). Unlike all of the other Sec^{-} strains examined here, the *sec14*^{ts}*-3* single-mutant strain exhibited levels of UPRE-*CYC-lacZ* expression indistinguishable from wild type when shifted to temperatures of 30° and 33° (data not shown), semipermissive temperatures at which we observed substantial UPRE induction in the *sec14*^{ts}*-3 cki1*Δ strain in a previous study (CHANG *et al.* 2002).

The mechanism of UPR activation involves splicing of the *HAC1* transcript by Ire1p, following activation of Ire1p by autophosphorylation (COX *et al.* 1993; MORI *et al.* 1993; COX and WALTER 1996). To determine whether *HAC1* splicing was elevated under secretory stress, Northern blot analysis was performed on RNA extracted from *sec13*^{ts}*-1* cells. As a control, wild-type cells were treated with 1 mM tunicamycin, which has been shown to elicit the UPR (COX and WALTER 1996). Untreated wild-type and *sec13*^{ts}*-1* cells grown at 25° contained only

the full-length (unspliced) *HAC1* transcript (Figure 1C). As expected, tunicamycin-treated wild-type cells contained two distinctive sizes of *HAC1* mRNA corresponding to unspliced (*HAC1^u^*) and spliced (*HAC1^i^*) mRNA (Figure 1C). *sec13^ts^-1* cells grown at the semipermissive temperature of 30°, in the absence of tunicamycin, likewise contained both the spliced and unspliced forms of *HAC1* transcript (Figure 1C), confirming that *HAC1* mRNA is actively spliced in *sec13^ts^-1* cells at 30°.

We observed a modest increase in the expression of β-galactosidase from the UPRE-*CYC-lacZ* construct after shifting wild-type cells to 36° (data not shown). Since changes in growth temperature are known to cause changes in metabolism, including alterations in the pattern of turnover of membrane lipids (Dowd *et al.* 2001), we examined activation of the UPR in strains treated with or without the fungal metabolite BFA at 30°. BFA blocks secretion, results in loss of COPI coats from the Golgi, and causes redistribution of Golgi enzymes to the ER (Lippincott-Schwartz *et al.* 1989; Klausner *et al.* 1992; Kreis *et al.* 1995; Sciaky *et al.* 1997; Seemann *et al.* 2000). Unlike mammalian cells, most *Saccharomyces cerevisiae* strains are insensitive to BFA. However, *erg6* mutants, which are defective in biosynthesis of ergosterol, exhibit sensitivity to BFA presumably due to increased membrane permeability (Graham *et al.* 1993; Shah and Klausner 1993; Vogel *et al.* 1993). As shown in Figure 1B, wild-type cells that are impermeable to BFA exhibited no UPRE induction in the presence of BFA at 30°. *erg6*Δ cells, however, exhibited high levels of UPRE-driven β-galactosidase expression at 30° in medium containing 25 and 50 mm BFA (Figure 1B), concentrations that do not inhibit growth of *erg6*Δ. Expression driven by the UPRE construct in the *erg6*Δ strain was elevated ~9-fold within 1 hr following exposure to 25 mm BFA (data not shown), compared to *erg6*Δ cells grown without BFA. After 2 hr of exposure to 25 mm BFA at 30°, UPRE expression was elevated ~15-fold in the *erg6*Δ strain (Figure 1B). Similar elevated levels of UPRE-driven β-galactosidase expression were observed at equivalent time points in *erg6*Δ cells grown in medium containing 50 mm BFA (Figure 1B).

***INO1* expression is reduced in Sec^−^ mutants experiencing secretory stress:** *INO1* expression was assayed in a selection of the Sec^−^ mutants as described in materials and methods using an *INO1-CYC-lacZ* reporter gene. All of the mutants tested exhibited reduced *INO1* expression compared to wild type when cultured at semipermissive growth temperatures in inositol-free (I^−^) medium (Figure 2A). This effect was most pronounced in the *sec13^ts^-1* and *sec16^ts^-2* strains, where a three- to fivefold reduction in *INO1-CYC-lacZ* expression was observed in cells grown in I^−^ medium shifted to 30° compared to the same cells grown continuously at 25° (Figure 2A). The *sec18^ts^-1*, *sec22^ts^-3*, and *sec21^ts^-1* mutants also exhibited levels of *INO1* expression that were 50% of wild type or lower, even at the permissive temperature of

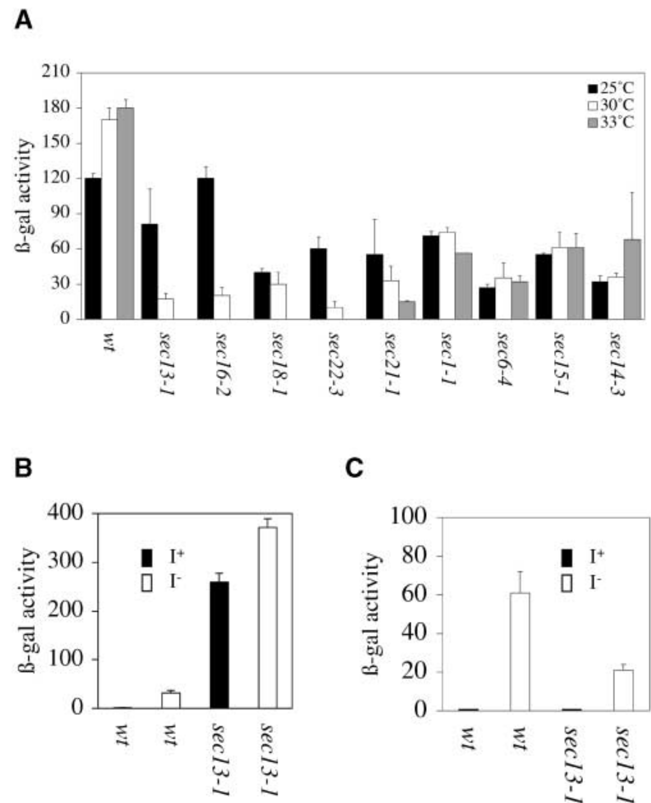

Figure 2.—*INO1* expression is reduced in Sec^−^ cells under conditions of secretory stress. (A) Sec^−^ strains were transformed with pJH359 (INO1-CYC-*lacZ*) as described in materials and methods and transformants were precultured at 25° to midlogarithmic phase of growth in I^−^ medium lacking uracil and shifted to the indicated temperatures. Samples were taken after 3 hr following the temperature shift and analyzed for β-galactosidase activity. β-Galactosidase activity is expressed as OD_{420}/min/ml. (B) *sec13-1* cells carrying pJC104 (UPRE-CYC-*lacZ*) were precultured at 30° to midlogarithmic phase of growth in I^+^ medium lacking uracil. Cells were filtered and resuspended in I^+^ or I^−^ medium, which also lacked uracil, at an OD_{600nm} = 0.5. The cultures were incubated for an additional 3 hr at 30° and then analyzed for β-galactosidase activity. (C) *sec13^ts^-1* cells carrying pJH359 (INO1-CYC-*lacZ*) were precultured at 30° to midlogarithmic phase of growth in I^+^ medium lacking uracil. Cells were filtered and resuspended in I^+^ or I^−^ medium lacking uracil at an OD_{600nm} = 0.5. Cultures were incubated at 30° for an additional 3 hr and analyzed for β-galactosidase activity.

25° (Figure 2A). A further reduction in *INO1-CYC-lacZ* expression was observed in these strains when they were shifted to the semipermissive temperatures of 30° or 33°. Of the strains tested (Figure 2), the *sec22^ts^-3* strain showed the lowest level of *INO1* expression at 30° (~10% of wild-type levels; Figure 2A). Somewhat reduced levels of *INO1* expression were also observed at all temperatures in the *sec1^ts^-1*, *sec6^ts^-4*, and *sec15^ts^-1* strains. In these cases, β-galactosidase activity from the *INO1-CYC-lacZ* reporter gene was reduced compared to wild type even at 25°, but was not significantly further reduced at 30° or 33°. In the *sec14^ts^-3* strain, expression

from the *INO1* reporter gene was reduced compared to wild type at 25° and 30°. Expression of *INO1-CYC-lacZ* increased somewhat after a shift to 33°, but did not reach the level of expression observed in the wild-type strain grown under comparable circumstances (Figure 2A).

Since the UPR is known to be activated under inositol-limiting conditions (Cox *et al.* 1997), we examined both UPRE-*CYC-lacZ* and *INO1-CYC-lacZ* expression in *sec13^{ts}-1* and wild-type cells shifted from inositol-containing (I^+) to inositol-free (I^-) medium. Wild-type and *sec13^{ts}-1* cells, transformed with *INO1-CYC-lacZ* or *UPRE-CYC-lacZ* reporter constructs, were first grown to logarithmic phase at 30° in I^+ medium and then shifted for 3 additional hours to I^- medium (Figure 2, B and C). Wild-type transformants treated in this fashion showed modest expression from the UPRE reporter gene following the shift to I^- medium at 30°, as well as derepression of the *INO1* reporter construct (Figure 2, B and C), consistent with the report of Cox *et al.* (1997).

In contrast to wild type, at 30°, *sec13^{ts}-1* cells exhibited high levels of β-galactosidase expression from the UPRE reporter gene whether inositol was present or not (Figure 2B). In fact, the level of UPRE expression in *sec13^{ts}-1* cells grown at 30° in I^+ medium, exceeded the level of UPRE expression in wild-type cells shifted to I^- medium by >8-fold. Following the shift to I^- medium, a further increase of ~30% in expression from the UPRE-*CYC-lacZ* reporter gene was observed in *sec13^{ts}-1* cells (Figure 2B). Overall, the level of β-galactosidase activity driven by the UPRE construct in *sec13^{ts}-1* cells grown at 30° in I^- medium exceeded that observed under the same conditions in wild-type cells by ~12-fold (Figure 2B). In contrast to the high level of UPRE expression observed in *sec13^{ts}-1* cells grown at 30° in I^+ medium (Figure 2B), *INO1-CYC-lacZ* expression in the *sec13^{ts}-1* cells at 30° was fully repressed when inositol was present (Figure 2C). When *sec13^{ts}-1* cells were shifted to I^- medium at 30°, *INO1-CYC-lacZ* derepression occurred, but the level of β-galactosidase activity achieved was only about one-third of the level seen in the wild-type strain (Figure 2C).

The *hac1*Δ and *ire1*Δ mutations exhibit partial synthetic lethality with mutations conferring defects in membrane trafficking: Sec$^-$ *ire1*Δ and Sec$^-$ *hac1*Δ strains were generated for each of the Sec$^-$ mutants used in this study and, in most cases, double-mutant progeny were found to have growth defects that were more extreme than those of the corresponding parental Sec$^-$ single mutant. Examples of Sec$^-$ *ire1*Δ double mutants, from each of the categories of Sec$^-$ mutants used for the temperature-shift experiments described above, are shown in Figure 3A. Sec$^-$ *hac1*Δ double-mutant strains, which are not shown, had phenotypes very similar to the corresponding Sec$^-$ *ire1*Δ strains. No change was detected in the growth pattern on YEPD plates in response to temperature of double mutants involving *sec1^{ts}-1* (Figure 3A) or *pep12*Δ with *ire1*Δ or *hac1*Δ, and slight, if any, difference was observed in the case of the double mutants involving *sec4^{ts}-8*, *sec14^{ts}-3*, and *sec15^{ts}-1*, as compared to the corresponding single mutants (data not shown). Double mutants involving *sec2^{ts}-59* and *sec21^{ts}-1* were not generated. In all other cases, the double mutants, Sec$^-$ *ire1*Δ or Sec$^-$ *hac1*Δ, failed to grow at a temperature ~2°–3° lower than that of the corresponding Sec$^-$ parent (Figure 3A; Sec$^-$ *hac1*Δ data not shown).

Mutations affecting COPII vesicle formation exhibited some of the strongest negative interactions with *ire1*Δ and *hac1*Δ. For example, the *sec13^{ts}-1 ire1*Δ and *sec13^{ts}-1 hac1*Δ strains failed to grow at a temperature of 30° or higher, while the restrictive temperature for the *sec13^{ts}-1* strain is 33° (Figure 3A). The *sec16^{ts}-2 ire1*Δ and *sec16^{ts}-2 hac1*Δ strains grew poorly even at 25°, while the *sec16^{ts}-2* parental strain was able to grow, although poorly, at temperatures up to 30° (Figure 3A). Double mutants simultaneously defective in the UPR and *sec12^{ts}-4* (Figure 3A) or *sec23^{ts}-1* (data not shown) also failed to grow at temperatures ~2° lower than the restrictive temperature for the corresponding parental Sec$^-$ strain. The *sec23^{ts}-1 ire1*Δ and *sec23^{ts}-1 hac1*Δ double mutants exhibited particularly poor growth and could grow only at 20° or lower on YEPD plates. They could not be cultured in liquid YEPD medium even at 20° and they failed to grow entirely on YEPD plates at 25° or higher. The *sec23^{ts}-1* parental strain, by contrast, was capable of normal growth on YEPD plates at temperatures up to 28° (data not shown). Double mutants carrying *ire1*Δ or *hac1*Δ in combination with mutations affecting vesicle docking and fusion, *sec17^{ts}-1* (Figure 3A), *sec18^{ts}-1*, or *sec22^{ts}-3* (data not shown), also failed to grow at temperatures 2°–3° lower than the minimum restrictive temperature of the corresponding parental Sec$^-$ mutant. Among the mutations that affect distal secretion from the Golgi to the plasma membrane, only *sec6^{ts}-4* exhibited a clear negative interaction with *ire1*Δ or *hac1*Δ. The *sec6^{ts}-4 ire1*Δ and *sec6^{ts}-4 hac1*Δ strains failed to grow at 33°, whereas the *sec6^{ts}-4* parental strain has a restrictive temperature of 35° (Figure 3A).

The *ire1*Δ and *hac1*Δ mutations were also crossed into the *erg6*Δ genetic background to assess their effect on BFA sensitivity. While growth of the *erg6*Δ parental strain was completely inhibited by the presence of 100 mM BFA, as previously reported (Graham *et al.* 1993; Shah and Klausner 1993; Vogel *et al.* 1993), and was unaffected by 25 or 35 mM BFA in YEPD medium (Figure 4), the *erg6*Δ *ire1*Δ and *erg6*Δ *hac1*Δ double-mutant strains were inhibited by 35 mM BFA (Figure 4). Thus, the presence of a UPR mutation results in an increased sensitivity of *erg6*Δ to BFA.

Growth of a subset of the Sec$^-$ strains was also examined at the semipermissive temperature of 30° on media containing or lacking inositol. As reported by Nikawa and Yamashita (1992), *ire1*Δ (Figure 3B) and *hac1*Δ strains (data not shown) exhibit somewhat "leaky" inositol auxotrophy (Ino$^-$ phenotype; Figure 3B). The

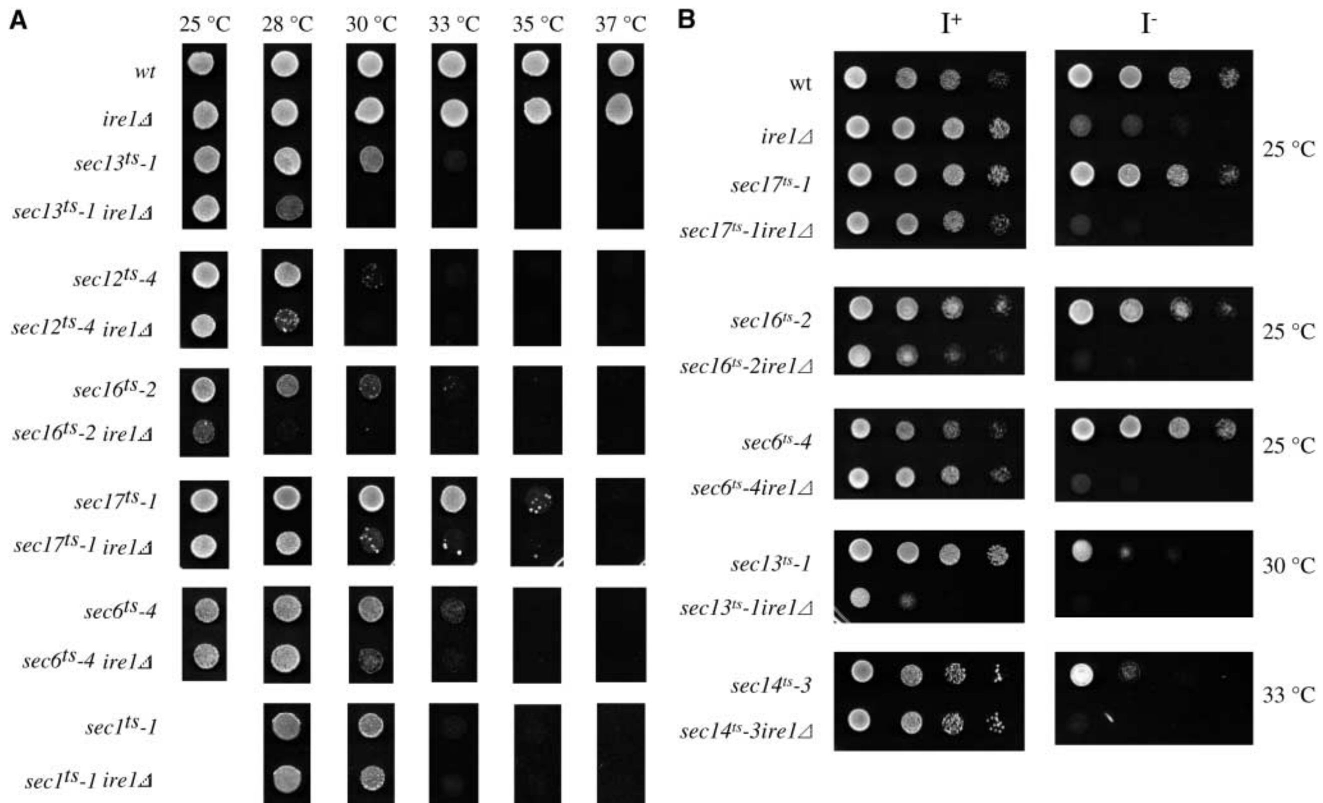

FIGURE 3.—(A) Genetic interactions between *ire1*Δ and Sec$^-$ mutations and Ino$^-$ phenotypes of Sec$^-$ Upr$^-$ mutants. The *ire1*Δ (either HCY405 or HCY423) strain was crossed to Sec$^-$ mutants (CKY46, RSY267, RSY269, and JBY318) to generate *Sec$^-$ ire1*Δ double-mutant strains. From each cross, four spore colonies from a single tetratype ascus were used to assess the growth phenotype. For simplicity, only growth of wild-type and *ire1*Δ spores generated from a cross of CKY46 and HCY405 is shown here, since all other wild-type and *ire1*Δ spores generated from other crosses showed similar growth phenotypes. HCY424 (wt), HCY423 (*ire1*Δ), HCY425 (*sec13*^{ts}*-1*), HCY422 (*sec13*^{ts}*-1 ire1*Δ), HCY280 (*sec12*^{ts}*-4*), HCY283 (*sec12*^{ts}*-4 ire1*Δ), HCY427 (*sec16*^{ts}*-2*), HCY428 (*sec16*^{ts}*-2 ire1*Δ), HCY437 (*sec17*^{ts}*-1*), HCY435 (*sec17*^{ts}*-1 ire1*Δ), HCY470 (*sec6*^{ts}*-4*), HCY473 (*sec6*^{ts}*-4 ire1*Δ), HCY495 (*sec1*^{ts}*-1*), and HCY496 (*sec1*^{ts}*-1 ire1*Δ) were precultured to midlogarithmic phase of growth at 20° in YEPD medium. The concentration of the cells was adjusted to OD_{600} = 0.7 with sterile dH_2O. Cells were initially diluted 1:100 using dH_2O followed by 1:10 serial dilutions. Four microliters of cells from each dilution were spotted on YEPD plates and allowed to grow at the designated temperatures for 3 days. (B) Strains generated as described in A, above, were precultured to midlogarithmic phase of growth at 25° in I$^+$ medium, washed twice, and spotted as a series of dilutions (1:10) on I$^+$ or I$^-$ medium and incubated at the temperatures shown. In the top, the phenotypes of four spore colonies from the cross of *ire1*Δ to *sec17-1* are shown. Below, the wild-type and *ire1*Δ colonies are omitted.

sec13-1 strain also has a leaky Ino$^-$ phenotype at its semipermissive temperature of 30° (Figure 3B) as reported by GILSTRING *et al.* (1999). The *sec14*^{ts}*-3* strain also grows more poorly in I$^-$ medium at the semipermissive temperature of 33° (Figure 3B), as reported by KEARNS *et al.* (1997) and CHANG *et al.* (2002). In a number of cases tested, the double mutants (*i.e.*, Sec$^-$ *ire1*Δ or Sec$^-$ *hac1*Δ) exhibited somewhat tighter Ino$^-$ phenotypes than either single-mutant parent did (Figure 3B). Growth of five such double mutants, *sec14* ^{ts}*-3, sec13*^{ts}*-1, sec6*^{ts}*-4, sec16*^{ts}*-2*, and *sec17*^{ts}*-1*, in combination with *ire1*Δ is shown in Figure 3B.

Constitutive activation of the UPR by expression of the *HAC1*^{i} gene rescues growth defects of some Sec$^-$ mutants: The *sec1*^{ts}*-1, sec4*^{ts}*-8, sec13*^{ts}*-1, sec13*^{ts}*-4, sec14*^{ts}*-3, sec15*^{ts}*-1*, and *sec22*^{ts}*-3* strains were transformed with the *HAC1*^{i} construct, lacking the intron sequence. The lack of the intron in *HAC1*^{i} results in constitutive activation of the UPR, even in *ire1*Δ strains (COX and WALTER 1996; KAWAHARA *et al.* 1997, 1998; MORI *et al.* 2000).

The *sec15*^{ts}*-1* and *sec14*^{ts}*-3* mutants transformed with pHAC1^{i} appeared to grow more poorly than the same strains carrying vector alone (data not shown), a result consistent with the reports of KAWAHARA *et al.* (1997) and MORI *et al.* (2000), who observed that wild-type strains transformed with pHAC1^{i} exhibited slower growth than controls. No change was observed in the growth of the *sec1*^{ts}*-1, sec22*^{ts}*-3*, or *sec4*^{ts}*-8* strains when transformed with pHAC1^{i} (data not shown). While the growth of the *sec1*^{ts}*-1* strain was not affected by transformation with pHAC1^{i} (data not shown), the *sec1*^{ts}*-1 ire1*Δ strain transformed with pHAC1^{i} was able to grow at 33° (Figure 5B), a temperature at which the *sec1*^{ts}*-1* single mutant is unable to grow (see Figure 3A). This result was not

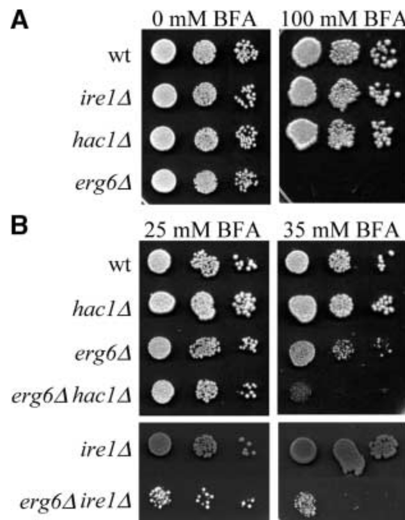

Figure 4.—Mutations in the UPR increase the sensitivity of *erg6*Δ cells to BFA. (A) As controls, HCY104 (wt), HCY105 (*ire1*Δ), HCY126 (*hac1*Δ), and HCY362 (*erg6*Δ) were tested for their ability to grow on YEPD medium containing 100 mm BFA. (B) Two sets of four spores containing the tetratype genotypes derived from a single tetrad (HCY462–HCY465 and HCY466–HCY469) were generated by crossing HCY362 (*erg6*Δ) to HCY401 (*ire1*Δ) or HCY030 (*hac1*Δ). Cells were precultured in YEPD medium at 30° to midlogarithmic phase (OD_{600} = 0.5) of growth, spotted as a series of dilutions (1:10) on YEPD plates containing 25 mm and 35 mm BFA, and allowed to grow at 30° for 4 days.

expected, since the *sec1^{ts}-1 ire1*Δ and *sec1^{ts}-1* strains had similar phenotypes at 33° (Figure 3).

The most dramatic effects of expression of pHAC1^{i} were obtained in the *sec13^{ts}-1* and *sec13^{ts}-4* strains. When transformed with pHAC1^{i}, the *sec13^{ts}-4* and *sec13^{ts}-4 hac1*Δ strains were able to grow at 37° (Figure 5A), a temperature that is restrictive for *sec13^{ts}-4*. The *sec13^{ts}-1* strain was also able to grow at 32° when transformed with pHAC1^{i} (data not shown), whereas *sec13^{ts}-1* will not grow above its semipermissive temperature of 30° when transformed with vector alone. *HAC1^{i}* was also able to rescue growth of *sec13^{ts}-1* in the absence of inositol at 30° and 32° (Figure 5C).

However, *HAC1^{i}* was not able to restore *sec13^{ts}-1* growth under any condition (with or without inositol) at temperatures of 33° or greater (Figure 5C), suggesting that *HAC1^{i}* expression cannot suppress complete loss of Sec13p function. To test this idea, a *sec13*Δ strain carrying the wild-type *SEC13* gene on plasmid pRS316 was transformed with pHAC1^{i} or an empty vector (YCp 50) and a plasmid "shuffling" experiment was performed, as described in materials and methods. When the transformants were forced to lose pSEC13, they failed to grow even when pHAC1^{i} was present (data not shown), indicating that expression of the *HAC1^{i}* gene cannot compensate for the total loss of Sec13p.

A functional UPR pathway improves the kinetics of transport to the vacuole in Sec^{-} mutants: To assess the effect of the UPR on membrane trafficking, we monitored the kinetics of processing in several Sec^{-} mutants, with and without a deletion in either *IRE1* or *HAC1.* These experiments were conducted at temperatures in which the UPR is activated in the corresponding Sec^{-} mutant, as determined in the experiments depicted in Figure 1A. In each case, the temperature selected was permissive or semipermissive for the corresponding Sec^{-} mutant, but restrictive in strains carrying the given Sec^{-} mutation in combination with *ire1*Δ or *hac1*Δ (*i.e.*, the specific Sec^{-}*ire1*Δ or Sec^{-}*hac1*Δ strains were incapable of sustained growth at the chosen temperature). The strains tested included *sec13^{ts}-1, sec13^{ts}-4, sec22^{ts}-3,* and *sec17^{ts}-1* in combination with *ire1*Δ or *hac1*Δ. Data are shown in Figure 5 for strains carrying *sec13^{ts}-1* and *sec13^{ts}-4* mutations. Cells were briefly shifted to the elevated temperature, as described in materials and methods, and the kinetics of CPY maturation were monitored in strains using pulse-chase analysis followed by CPY immunoprecipitation and SDS-PAGE analysis followed by image analysis of the autoradiograms (Figure 6).

In the wild-type strain at 30°, the 67-kD ER form of CPY (Figure 6, p1) was rapidly converted to the 69-kD Golgi form (Figure 6, p2), which is proteolytically cleaved in the vacuole to the mature 61-kD form (Figure 6, mCPY). Thus, the appearance of mCPY signals the delivery of CPY to the vacuole (Stevens *et al.* 1982). The kinetics of CPY processing in the *sec13^{ts}-1* strain at 30°, which is a semipermissive temperature for this mutant allele of *SEC13* (Figure 3A), were somewhat delayed compared to wild type (Figure 6A). The delay is most visible at the 10-min time point. At 10 min in *sec13^{ts}-1*, about one-third of labeled CPY was recovered in mCPY, compared to about two-thirds in wild type, and the remaining label in CPY was distributed between the p1 and p2 forms (Figure 6, A and C). After 20 min in the *sec13^{ts}-1* strain, most of the labeled CPY was recovered in mCPY (Figure 6, A and C). By 30 min, there was very little difference between *sec13^{ts}-1* and wild type in the amount of m relative to the p2 Golgi form, but the p1 ER form was observed in the *sec13^{ts}-1* strain, even after 30 min. This observation is consistent with the reported ER to Golgi vesicular transport defect of *sec13* mutants (Novick *et al.* 1980, 1981). In the *sec13^{ts}-1 ire1*Δ strain, which is not capable of sustained growth at 30°, the kinetics of CPY processing at 30° were slowed still further as compared to both the wild-type and *sec13^{ts}-1* strains at 30°. In this strain, after 10 min, less than one-quarter of labeled CPY was present in mCPY (Figure 6, A and C). Comparable slowing in the appearance of mCPY was observed in the *sec17^{ts}-1 ire1*Δ and *sec22^{ts}-3 ire1*Δ strains relative to the *sec17^{ts}-1* and *sec22^{ts}-3* strains, respectively (data not shown), suggesting that slowing of CPY processing is not unique to the *sec13^{ts}-1 ire1*Δ strain. Moreover, the kinetics of processing in an *ire1*Δ strain were identical to wild type (data not

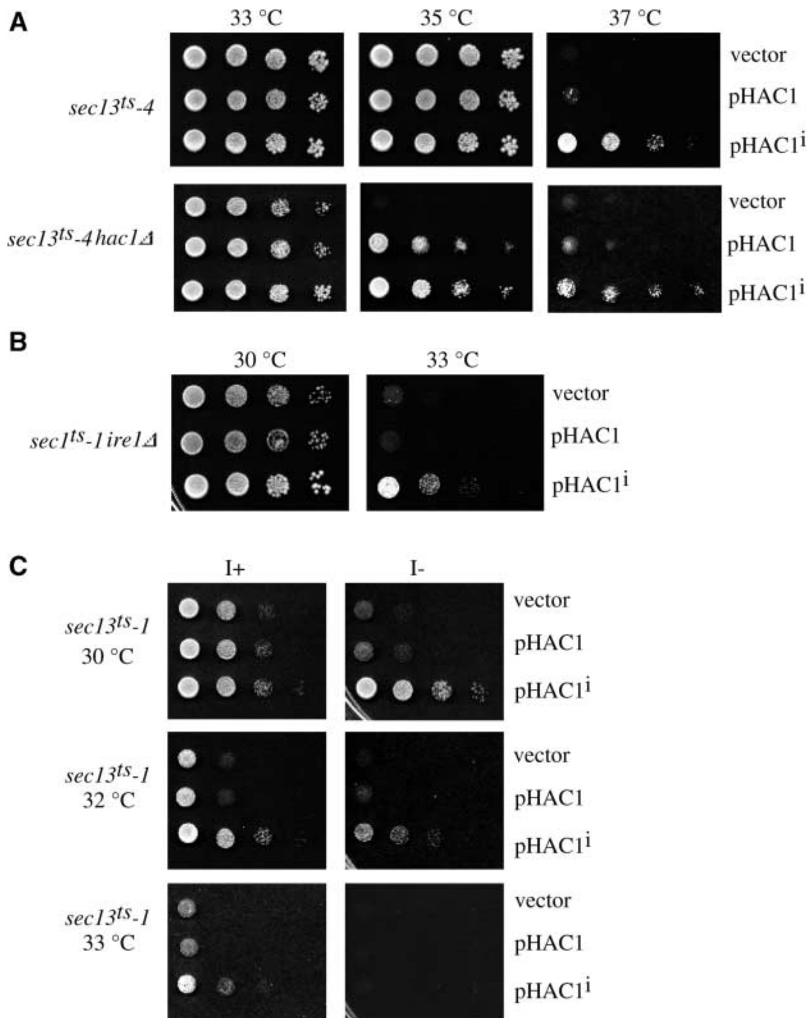

Figure 5.—Expression of *HAC1*^{i} partially suppresses the growth defect (*ts*) and Ino^{-} phenotypes of some Sec^{-} strains. (A) Growth at different temperatures of HCY180 (*sec13*^{ts}*-4*) and HCY178 (*sec13*^{ts}*-4 hac1*Δ) cells transformed with pYC50 (empty vector), pHAC1 (wild-type *HAC1*), and pHAC1^{i} (intronless *HAC1*) is shown. (B) Growth at different temperatures of HCY496 (*sec1*^{ts}*-1 ire1*Δ) cells transformed with pYC50, pHAC1, and pHAC1^{i}. Transformants in both A and B were precultured to midlogarithmic phase of growth (OD_{600} = 0.5) at 20° in medium lacking leucine, spotted as a 10-fold dilution series on leucine-free plates, and incubated at the indicated temperatures for 4 days. (C) Effect of pHAC1 and pHAC1^{i} on the Ino^{-} and *ts* phenotypes of *sec13-1*. *sec13-1* cells were transformed with pYC50 (empty vector), pHAC1 (wild-type *HAC1*), and pHAC1^{i} (intronless *HAC1*). Transformants were precultured to midlogarithmic phase of growth (OD_{600} = 0.5) at 20° in medium lacking leucine, spotted as a 10-fold dilution series on leucine-free plates containing (I^{+}) or lacking (I^{-}) inositol, and incubated for 3 days at the indicated temperature.

shown), indicating that the UPR pathway defect does not appreciably affect normal membrane trafficking in the absence of secretory stress.

We also measured the time course of CPY processing in the *sec13*^{ts}*-4 hac1*Δ strain expressing the *HAC1*^{i} gene from a centromeric plasmid and compared it to processing in the same strain transformed with an empty vector (Figure 6B). Transformants were briefly shifted to 35°, conditions in which the *HAC1*^{i} gene product rescues the growth defect of *sec13*^{ts}*-4 hac1*Δ strains (Figure 5A). Transformants were then subjected to pulse-chase analysis as described above. In the *sec13*^{ts}*-4 hac1*Δ strain transformed with an empty vector, most of the labeled CPY remained in the ER (p1) form throughout the time course (Figure 6, B and C). However, in the *sec13*^{ts}*-4 hac1*Δ strain transformed with the *HAC1*^{i} gene, the kinetics of CPY processing were markedly faster than in the control transformed with vector alone (Figure 6, B and C). After 10 min, about one-third of the labeled CPY was present in mCPY compared to ∼ <20% in transformants containing vector alone (Figure 6, B and C). After 30 min, less than half of labeled CPY was processed to m in the transformants carrying vector alone, whereas in *sec13*^{ts}*-4 hac1*Δ cells transformed with pHAC1^{i}, ∼80% of the label was present in mCPY after 30 min.

DISCUSSION

In this study, we have documented a functional interaction between the UPR and the secretory pathway. The UPR is activated in Sec^{-} mutants defective in events extending from ER vesicle trafficking to distal secretion and in Pep^{-} mutants defective in vacuolar targeting (Figure 1). The UPR is also activated in *erg6*Δ cells challenged with BFA (Figure 1). Moreover, our results indicate that activation of the UPR plays a functional role during secretory stress, facilitating protein trafficking in cells experiencing partial impairment in secretory function. This conclusion is supported by the partial synthetic lethality of the *ire1*Δ and *hac1*Δ mutations in combination with a large majority of Sec^{-} mutants tested in this study (Figure 3), as well as the partial suppression of certain Sec^{-} phenotypes by *HAC1*^{i} (Figure 5). The slowing of CPY processing in Sec^{-} strains carrying a UPR mutation and the rescue of these defects

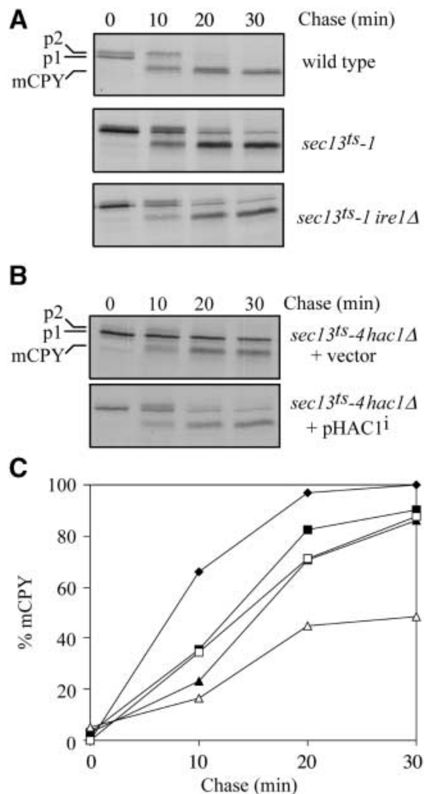

FIGURE 6.—Comparison of processing in *sec13* mutant strains when the UPR is absent or constitutively active. (A) The rate of conversion of to its mature form was determined by pulse labeling HCY424 (wt), HCY425 (*sec13^{ts}-1*), and HCY422 (*sec13^{ts}-1 ire1*Δ) cells for 5 min at 30° with [^{35}S]methionine/cysteine, chasing with cold amino acids at 30° for the indicated times, and immunoprecipitating with anti-antibodies. ER-glycosylated (p1), Golgi-modified (p2) precursors, and vacuolar mature form (mCPY) are indicated. (B) The rate of conversion of in HCY178 (*sec13^{ts}-4 hac1*Δ) transformants containing empty vector or expressing the *HAC1^{i}* gene from a centromeric plasmid was measured by pulse labeling cells at 35° and chasing with cold amino acids for the indicated times. Processing of was analyzed following immunoprecipitation and SDS-PAGE. (C) Plotted percentages of mature at indicated times determined from autoradiograms shown in A and B, as described in MATERIALS AND METHODS. Solid diamonds, wild type; solid squares, *sec13ts-1*; solid triangles, *sec13ts-1 ire1*Δ; open diamonds, *sec13^{ts}-4 hac1*Δ + vector; open squares, *sec13^{ts}-4 hac1*Δ + pHAC1^{i}.

by *HAC1^{i}* provide direct confirmation that UPR plays a role in facilitating protein processing under these conditions (Figure 6).

However, under conditions of secretory stress created by elevating Sec^{-} mutants to a semipermissive temperature, the observed activation of the UPR does not result in elevated *INO1* expression compared to wild type, even when inositol is absent. Indeed, under conditions of secretory stress, in Sec^{-} cells exposed to semipermissive growth conditions, *INO1* expression is actually reduced compared to wild type (Figure 2) and some strains, such as *sec13^{ts}-1* and *sec14^{ts}-3* grown at their semipermissive temperatures, actually require inositol for growth (Figure 3B). Thus, it appears that the activation of the UPR, which occurs under conditions of secretory stress, is not correlated with elevated *INO1* expression.

The role of the UPR and the secretory pathway in expression and regulation of the *INO1* gene: *INO1* is one of a large number of coregulated genes, which contain the promoter element UAS_{INO} and respond to the availability of inositol in the growth medium (CARMAN and HENRY 1999). While the mechanism by which the cell regulates *INO1* in response to inositol and other precursors of lipid metabolism has not been fully elucidated, it is known that the Ino2p and Ino4p transcription factors bind to UAS_{INO} to activate transcription (CARMAN and HENRY 1989; LOPES *et al.* 1991; AMBROZIAK and HENRY 1994; SCHWANK *et al.* 1995; GREENBERG and LOPES 1996). Furthermore, the negative regulator, Opi1p, is required for repression of UAS_{INO}-containing genes in response to inositol (WHITE *et al.* 1991). Recent evidence suggests that changes in the pattern of membrane phospholipid synthesis, produced in response to the incorporation of exogenous inositol, result in translocation of the negative regulator, Opi1p, to the nucleus from the endoplasmic reticulum and that Opi1p translocation coincides with repression of *INO1* (LOEWEN *et al.* 2004).

COX *et al.* (1997) suggested that activation of *INO1* transcription in the absence of inositol might be linked to activation of the UPR signal transduction pathway. In support of this hypothesis, COX *et al.* (1997) showed that the UPR is activated in wild-type cells growing in inositol-free medium and that deletion of *OPI1* suppressed the Ino^{-} phenotype of *ire1*Δ and *hac1*Δ mutants. Previously, consistent with the hypothesis of Cox *et al.*, we reported that expression of both UPR and *INO1* is elevated in *sec14-3^{ts}* strains carrying the *cki1*Δ suppressor (CHANG *et al.* 2002). However, deletion of *HAC1* or *IRE1* did not eliminate overexpression of *INO1* in *sec14^{ts} cki1*Δ cells (CHANG *et al.* 2002), suggesting that activation of the UPR, while correlated with *INO1* expression, is not obligatory for *INO1* activation.

It has been assumed that the Ino^{-} phenotype of UPR mutants, such as *hac1*Δ and *ire1*Δ, is due to the inability to maintain wild-type *INO1* expression levels in the absence of UPR activation (COX *et al.* 1997; CHANG *et al.* 2002). Another possibility is that inositol deprivation results in a stress condition that elicits UPR activation and that UPR activation under these circumstances is essential in much the same way that it is in wild-type cells experiencing stress due to the buildup of unfolded proteins following exposure to tunicamycin. We propose that providing inositol to UPR mutants under such circumstances alleviates the underlying stress condition that necessitates UPR activation for survival, thus explaining the Ino^{-} phenotype. Since inositol limitation (COX *et al.* 1997; CHANG *et al.* 2002) and secretory stress (Figure 1) both result in UPR activation, we questioned whether these two stress conditions might have additive effects upon the level of UPRE and/or *INO1* expression.

Indeed, shifting the *sec13*^{ts}*-1* strain to inositol-free medium at the semipermissive temperature of 30° resulted in an increase of ~30% in UPRE expression over the already elevated levels seen in this strain at 30° in the presence of inositol (Figure 2).

Inositol limitation and secretory stress, thus, affect UPR activation in an additive or synergistic fashion. In Sec$^-$ cells growing near their restrictive temperatures, the additive stress caused by lack of inositol may, in some cases, exceed the stress tolerance of even those cells with an intact UPR. Provision of inositol under such circumstances would reduce the stress that results in UPR induction, potentially explaining the conditional Ino$^-$ phenotype of *sec13*^{ts}*-1* cells near their restrictive temperature. Consistent with this idea, transformation of *sec13*^{ts}*-1* with *HAC1*^{i} permits growth at temperatures up to 32° with or without inositol (Figure 5), whereas the parent *sec13*^{ts}*-1* strain transformed with the vector grows well only up to ~30° and only if inositol is present (Figure 5).

While inositol limitation and secretory stress had an additive effect upon UPRE expression levels, no correlated additive effect on *INO1-CYC-lacZ* expression was observed in *sec13*^{ts}*-1* cells grown in the absence of inositol at semipermissive temperatures (Figure 2A). To the contrary, in *sec13*^{ts}*-1* cells shifted to 30° in the absence of inositol, *INO1-CYC-lacZ* expression was reduced to less than one-half of the level observed in the wild-type control grown under identical conditions (Figure 2C). Similar reductions in *INO1-CYC-lacZ* expression levels, relative to the wild-type control were observed in all of the Sec$^-$ mutants assayed after growth in inositol-free media at semipermissive conditions (Figure 2A). The absence of a correlation between UPRE and *INO1* expression was even more apparent in *sec13*^{ts}*-1* cells grown at 30° in the presence of inositol (compare Figure 2B and 2C). Under these growth conditions, UPRE expression was greatly elevated in *sec13*^{ts}*-1* cells (Figure 2B). Yet, *INO1-lacZ* expression in the *sec13*^{ts}*-1* strain at 30° was fully repressed when inositol was present (Figure 2C). Clearly, activation of the UPR during secretory stress does not result in activation of *INO1* when inositol is present.

The *sec14-3*^{ts} mutation, unlike the other Sec$^-$ mutations analyzed here, does not affect an immediate component of the secretory apparatus itself. Rather, Sec14p is a lipid transfer protein that binds both phosphatidylinositol and phosphatidylcholine (Aitken *et al.* 1990). s*ec14* mutations result in a wide range of changes in both lipid metabolism and membrane trafficking (Novick *et al.* 1980, 1981; Bankaitis *et al.* 1989; Aitken *et al.* 1990; Cleves *et al.* 1991; Kearns *et al.* 1997; Patton-Vogt *et al.* 1997; Henry and Patton-Vogt 1998; Sreenivas *et al.* 1998; Xie *et al.* 1998). Surprisingly, despite the wide range of Sec$^-$ mutants that exhibited UPRE activation at their semipermissive temperatures, we did not observe this effect in the *sec14-3*^{ts} single mutant. The failure to observe UPRE activation in the *sec14-3*^{ts} strain at the semipermissive temperatures of 30° and 33° was very surprising given that high levels of UPRE activation were previously observed at both 30° and 37° (Chang *et al.* 2002) in *sec14-3*^{ts} strains carrying the bypass suppressor, *cki1*Δ. Inada and Guthrie (2004), however, recently reported active splicing of *HAC1* mRNA, indicative of UPR activation, in the *sec14-3*^{ts} mutant shifted to its restrictive temperature. Thus, it is possible that we failed to observe UPR activation in the *sec14-3*^{ts} mutant because we used a less direct assay (*i.e.*, a UPRE reporter construct *vs.* *HAC1* mRNA splicing) and different growth conditions (*i.e.*, semipermissive temperature *vs.* a transient shift to the restrictive condition) than did Inada and Guthrie (2004). At 30° and 37°, the *sec14-3*^{ts} *cki1*Δ double mutant exhibits high levels of *INO1* expression and inositol prototrophy and, in fact, overproduces and excretes inositol into the growth medium (Patton-Vogt *et al.* 1997; Chang *et al.* 2002). In contrast, the *sec14-3*^{ts} single mutant exhibits inositol auxotrophy and lowered *INO1* expression at the semipermissive temperature of 33°. The reduction in *INO1* expression in *sec14*^{ts}*-3* under semipermissive growth conditions was similar to that in other Sec$^-$ strains studied here, suggesting that lowered *INO1* expression is a general response to impairment of secretory function. The elevated *INO1* expression that we reported previously in the *sec14*^{ts}*-3 cki1*Δ strain, and other *sec14* strains carrying bypass suppressors affecting the CDP-choline pathway for phosphatidylcholine synthesis, is presumably a consequence of the specific mechanism of suppression (Patton-Vogt *et al.* 1997; Henry and Patton-Vogt 1998; Chang *et al.* 2002).

Regardless of the explanation for the general lack of correlation between UPR activation and *INO1* expression in Sec$^-$ mutants, it is clear that *INO1* expression is reduced in Sec$^-$ mutants experiencing secretory stress. The inhibitory effect of secretory stress on transcription of *INO1* is similar to the effect of secretory stress on transcription of rRNA and ribosomal protein genes. Mizuta and Warner (1994) showed that the function of the entire secretory pathway is essential for ribosomal synthesis. Nierras and Warner (1999) subsequently demonstrated that rRNA transcription and ribosomal protein synthesis are slowed in cells undergoing secretory stress and that this response is not transduced by the UPR. Rather, they concluded that the effect on rRNA and ribosomal proteins genes in cells with secretory defects may be controlled by a mechanism involving the protein kinase C signal transduction pathway related to membrane stress. In similar fashion, under conditions of secretory stress, signals other than those generated by the UPR may take precedence in controlling expression of *INO1* and other UAS_{INO}-containing genes. *INO1* expression is known to be influenced by growth phase and nutrient availability Griac and Henry (1999) and the glucose response pathway has also been shown to influence the levels of *INO1* expression (Ouyang *et*

al. 1999; Shirra and Arndt 1999; Shirra *et al.* 2001). For these reasons, we are currently examining the relative roles of several other signal transduction pathways in transducing signals from the secretory pathway to the regulatory apparatus controlling *INO1* transcription.

The UPR plays a functional role in cells experiencing secretory stress: The idea that the UPR might play a functional role in exit from the ER and/or be induced by any slowing of ER-specific steps seems quite logical given that the UPR is known to regulate gene expression in response to stress in the ER. Several previous studies have suggested that the UPR might be activated in mutants having defects in specific early steps in membrane trafficking in yeast. For example, Rose *et al.* (1989) detected an increased level of *KAR2* mRNA in the *sec18*^{ts}*-1* mutant, which is defective in vesicle fusion and exit from the ER and Semenza *et al.* (1990) reported secretion of Kar2p (BiP) from various Sec^{-} mutants, including *sec22*^{ts}*-3*, *sec17*^{ts}*-1*, *sec20*^{ts}*-1*, and *sec18*^{ts}*-1*, at their permissive temperatures. Belden and Barlowe (2001) demonstrated that the secretion of Kar2p in *sec22*^{ts}*-3* cells, reported by Semenza *et al.*, is associated with strong UPR activation. Since Sec22p may function in retrograde transport of proteins from the Golgi to the ER, Belden and Barlowe (2001) speculated that accumulation of secretory proteins in the ER in *sec22*^{ts}*-3* cells at permissive temperatures could lead to proliferation of the ER, thereby activating the UPR.

The experiments we report here using BFA-treated *erg6*Δ cells and a wide range of secretory mutants suggest that the UPR can be triggered by secretory stress induced not only by mutations affecting exit from the ER, but also by mutations affecting a number of compartments of the secretory pathway. In the case of mutants such as *sec12*^{ts}*-4*, *sec13*^{ts}*-1*, *sec13*^{ts}*-4*, *sec16*^{ts}*-2*, and *sec23*^{ts}*-1*, which have defects in COPII vesicle formation, and in *erg6*Δ cells treated with BFA, UPR activation might be triggered by disruption of the ER organization and accumulation of secretory cargo proteins as suggested by Belden and Barlowe. However, activation of the UPR in mutants defective in vacuolar targeting (*pep12*Δ) and the Sec6p complex (Figure 1A) indicates that secretory stress induced in compartments distal to the ER is also able to elicit the UPR through Ire1p, a kinase believed to be localized exclusively in the ER (Cox *et al.* 1993). In a related recent report, Leber *et al.* (2004) have shown that ER-distal stress boosts *HAC1* mRNA abundance.

Travers *et al.* (2000) demonstrated that activation of the UPR affects expression of genes controlling a broad array of ER and secretory functions including ER-associated protein degradation (ERAD), ER-to-Golgi transport, Golgi-to-ER retrieval, vacuolar targeting, distal secretion, and cell wall biogenesis. Travers *et al.* also showed that the UPR was activated in ERAD mutants and that *ire1*Δ and *hac1*Δ mutants exhibit synthetic lethality with ERAD mutations. Moreover, Caldwell *et al.* (2001) and Vashist *et al.* (2001) demonstrated that degradation of soluble substrates by ERAD requires ER-Golgi transport, suggesting a functional relationship involving ERAD and membrane trafficking from the ER.

We have demonstrated similar synthetic lethality involving UPR and secretory mutations. In most cases, Sec^{-} Upr^{-} double mutants had more severe growth phenotypes than the corresponding Sec^{-} parent. These results are consistent with the hypothesis that UPR induction provides protection to cells experiencing stress resulting from partial impairment of membrane trafficking and/or inositol limitation. Also consistent with this hypothesis is the partial suppression (*i.e.*, elevation of the restrictive temperature by several degrees and alleviation of the Ino^{-} phenotype) of *sec13*^{ts}*-1* by transformation with *HAC1*^{i} (Figure 5). Consistent with the results reported here, Higashio and Kohno (2002) and Sato *et al.* (2002) recently reported partial suppression by transformation with pHAC1^{i} of growth defects of the *sec24*^{ts}*-20* and *sec12*^{ts}*-4* mutants, respectively, which are defective in COPII vesicle formation. Furthermore, processing of was enhanced at the semipermissive temperature in Sec^{-} cells retaining an active UPR pathway or carrying *HAC1*^{i}, as compared to Sec^{-} cells carrying a UPR mutation (Figure 6). Thus, the UPR not only is activated under secretory stress, but also plays a role in facilitating membrane trafficking and cell survival under these conditions.

By what mechanism might secretory stress signals generated in membrane compartments, other than the ER, result in UPR activation? One possibility is that disruption of post-Golgi trafficking perturbs the balance of anterograde and retrograde membrane transport pathways between the ER and Golgi by affecting the rate and/or efficiency of protein sorting at the Golgi. It has been suggested that for protein sorting in late-Golgi compartments, anterograde and retrograde transport pathways coexist and compete with each other (Cole *et al.* 1998). In mammalian cells, it has been shown that Golgi resident proteins continually cycle through the ER (Cole *et al.* 1998; Miles *et al.* 2001; Ward *et al.* 2001). It has been suggested that one function for this recycling is to allow Golgi residents to be periodically surveyed by the protein-folding machinery in the ER where they could be either refolded or degraded through the ERAD pathway (Cole *et al.* 1998; Ng *et al.* 2000). When anterograde pathways are blocked or slowed, retrograde pathways predominate, leading to a return of secretory and resident proteins to the ER through normal recycling pathways. The ER might sense the accumulation of these proteins, which, in turn, might account for activation of the UPR. Alternatively, there may be as yet unidentified signaling that occurs between compartments of the secretory pathway serving to coordinate the overall flow of materials between compartments. Clearly, whatever the mechanism, the UPR is activated under secretory stress generated in a number of membrane compart-

ments and its activation provides functional protection, allowing Sec^{-} cells to grow at temperatures that are lethal in the absence of a functional UPR (Figure 3).

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