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The Unfolded Protein Response Coordinates the Production of Endoplasmic Reticulum Protein and Endoplasmic Reticulum Membrane

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The endoplasmic reticulum (ER) is a multifunctional organelle responsible for production of both lumenal and membrane components of secretory pathway compartments. Secretory proteins are folded, processed, and sorted in the ER lumen and lipid synthesis occurs on the ER membrane itself. In the yeast *Saccharomyces cerevisiae*, synthesis of ER components is highly regulated: the ER-resident proteins by the unfolded protein response and membrane lipid synthesis by the inositol response. We demonstrate that these two responses are intimately linked, forming different branches of the same pathway. Furthermore, we present evidence indicating that this coordinate regulation plays a role in ER biogenesis.

INTRODUCTION

The cytoplasm of eukaryotic cells is subdivided into functionally distinct membrane-bounded compartments, or organelles, that allow cells to carry out a multitude of specialized functions required for normal growth. Furthermore, the amount and composition of any organelle is altered in response to the changing needs of the cell (for review, see Nunnari and Walter, 1996). For example, as a B lymphocyte differentiates into a plasma cell, the endoplasmic reticulum (ER) expands to accommodate the increased flux of secretory proteins through the organelle (Wiest *et al.*, 1990). Likewise, during repeated muscle contraction, the relative number of mitochondria increase to satisfy the demand for more energy (Hood *et al.*, 1994).

Any increase in the amount of a particular organelle requires that the cell coordinately increases synthesis of the protein and lipid components that comprise the organelle. In many cases, regulation of organelle synthesis is achieved by transcriptional networks that respond to a change in demand for the function of a particular organelle, leading to altered expression of genes encoding organellar proteins (Nunnari and Walter, 1996 and references therein). However, little is known about how the necessary regulation of lipid components is coordinated.

Synthesis of lumenal ER proteins is regulated by the unfolded protein response (UPR),[1] a signal transduction cascade that allows eukaryotic cells to respond to changing conditions in the ER. The accumulation of unfolded proteins in the ER leads to increased transcription of genes encoding ER-localized chaperones (Lee, 1987; Kozutsumi *et al.*, 1988). Although present in all of the eukaryotic cells examined, the UPR is best understood in the yeast *Saccharomyces cerevisiae*. Experimentally, the UPR can be activated in yeast by blocking glycosylation with drugs such as tunicamycin (Tm) or by preventing disulfide-bond formation with reducing agents such as 2-mercaptoethanol. Genes known to be up-regulated by such treatments include *KAR2* (encoding Kar2p or BiP), *PDI1* (encoding protein disulfide isomerase), *EUG1* (encoding a PDI-like protein), and *FKB2* (encoding a peptidyl-prolyl *cis-trans* isomerase). These genes encode proteins that help catalyze the correct folding of proteins

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[1] Abbreviations used: Tm, tunicamycin; UPR, unfolded protein response; UPRE, UPR element.

in the ER (reviewed in Gething and Sambrook, 1992; Shamu *et al.*, 1994; Sweet, 1993).

To date, three components of the UPR have been identified. One of these is the transmembrane kinase Ire1p, encoded by the nonessential gene *IRE1*. Cells in which *IRE1* has been deleted do not increase transcription of ER-resident proteins in response to induction of the UPR (Cox *et al.*, 1993; Mori *et al.*, 1993). Ire1p is located in the ER and/or inner nuclear membrane and is likely to be the component that transmits the unfolded protein signal across the membrane. Its N-terminal domain lies in the lumen of the ER, where it presumably detects the accumulation of unfolded proteins, and its kinase domain lies in the cytoplasm (or nucleus), where it is proposed to be responsible for transmitting the unfolded protein signal to the appropriate downstream component(s). Ire1p, like most receptor transmembrane kinases, is thought to be activated by oligomerization and phosphorylation by neighboring Ire1p molecules (Shamu and Walter, 1996).

Hac1p, encoded by the *HAC1* gene, is a bZIP transcription factor that acts downstream of Ire1p in the UPR and binds to a 23-bp UPR element (UPRE) in the promoter of UPR-regulated genes (Cox and Walter, 1996; Nikawa *et al.*, 1996; Mori *et al.*, 1996; Shamu, 1997). Hac1p is detectable in UPR-activated cells only, and its level is controlled by regulated splicing of its mRNA. The spliced *HAC1* mRNA encodes a form of Hac1p that is more efficiently synthesized and/or more stable than the protein encoded by the unspliced message (Cox and Walter, 1996). Interestingly, *HAC1* mRNA bypasses spliceosome-mediated processing and is spliced in a nonconventional manner involving tRNA ligase, which thus constitutes another component of the UPR pathway (Sidrauski *et al.*, 1996). A specific allele, *rlg1–100*, of the essential tRNA ligase gene, has no detectable tRNA-splicing defect but completely blocks *HAC1* mRNA splicing. Like *ire1* and *hac1* mutants, *rlg1–100* mutants are completely blocked for the UPR.

The ER membrane is the major site of lipid synthesis in the cell and, like ER lumenal protein synthesis, ER membrane production is regulated by a transcriptional signaling pathway. In yeast the ER membrane has a high glycerophospholipid content, with a particularly high proportion of phosphatidylinositol (PI). Both the transcription of genes encoding enzymes required for synthesis of phospholipids and the activities of these enzymes are regulated primarily by the intracellular concentration of free inositol, a precursor in the synthesis of PI (Greenberg and Lopes, 1996). When intracellular inositol levels fall, the transcription of a wide variety of genes that are required for de novo phospholipid synthesis including *INO1* (encoding inositol-1-phosphate synthase), *CHO1* (encoding phosphatidylserine synthase), and *OPI3* (encoding phospholipid methyltransferase required for de novo phosphatidylcholine synthesis) is induced (Hirsch and Henry, 1986; Bailis *et al.*, 1987; Kodaki *et al.*, 1991). These genes contain at least one copy of a specific 10-bp DNA sequence in their promoter region called the UAS_{INO} that is necessary and sufficient for the inositol response (for review, see Carman and Henry, 1989).

How information about the inositol concentration in the cell is sensed and transmitted to the UAS_{INO} is unknown; however, the transcription factors affecting the activity of UAS_{INO} have been identified. At high inositol concentrations, transcription of phospholipid biosynthetic genes is repressed by the *OPI1* gene product, Opi1p. *opi1* mutant cells constitutively express all of the genes regulated by inositol. The sequence of Opi1p suggests that it is a transcription factor, as it contains both a leucine zipper domain and a glutamine rich region, but it is not known whether Opi1p binds to DNA directly (White *et al.*, 1991). Upon inositol starvation, Opi1p is inactivated allowing the heterodimeric transcriptional activator Ino2p/Ino4p to bind the UAS_{INO} and activate transcription. *ino2* or *ino4* mutant strains cannot transcribe genes controlled by the UAS_{INO}, including *INO1*, and thus are inositol auxotrophs (Hirsch and Henry, 1986).

Interestingly, *ire1*, *hac1*, and *rlg1–100* mutants also do not grow in the absence of inositol in the growth medium (Nikawa and Yamashita, 1992; Sidrauski *et al.*, 1996). Models to explain the link between inositol metabolism and the UPR suggest the intriguing possibility that the de novo synthesis of phospholipids and the up-regulation of ER content proteins are linked (Cox *et al.*, 1993; Mori *et al.*, 1993). In this article we demonstrate that the UPR-dependent synthesis of ER lumenal components and inositol concentration-dependent synthesis of ER membrane components are intimately connected. Furthermore, we provide evidence that coupling of these two pathways may serve to coordinate expansion of both the membrane and lumenal components of the ER.

MATERIALS AND METHODS

Media and General Methods

YPD (complete) and synthetic minimal media are described by Sherman (1991). Minimal medium lacking inositol was made as described by Culbertson and Henry (1975). When added, inositol (myo-inositol, Sigma, St. Louis, MO) was at a final concentration of 50 μg/ml. When added, Tm (Boehringer Mannheim, Indianapolis, IN) was at a final concentration of 1 μg/ml. Yeast transformations were performed by lithium acetate procedures (Ito *et al.*, 1983; Elble, 1992).

Yeast Strains and Plasmids

Yeast strains used in this study are listed in Table 1. The 2 μ-*INO1* plasmid pJH318 was a kind gift from Susan Henry (Carnegie Mellon University, Pittsburgh, PA). pRC1 was constructed by insertion of a

Table 1. Yeast strains

Strain	Genotype	Source/Reference
JC104	*MATα; leu2-3,-112::LEU-UPRE-lacZ; his3-11,-15::HIS3-UPRE-lacZ; ura3-1; trp1-1; ade2-1; can1-100*	Cox *et al.* (1993)
CS173	Same as JC104 except *ire1::URA3*	Cox *et al.* (1993)
JC132	*MATa; ire1::URA3; his3-11,-15::HIS3-UPRE-lacZ; leu2-3,112; ura3-1; trp1-1; ade2-1; can1-100*	This study
JC140	*MATa; ire1::URA3; opi1::LEU2; his3-11,-15::HIS3-UPRE-lacZ; ura3; trp1-1; leu2*	This study
JC193	*MATa; ino1::HIS3; his3-Δ200; leu2-Δ1; trp1-Δ63; ura3-52*	Susan Henry
JC198	*MATα; ino4::LEU2; leu2; his3; trp1; ura3*	Susan Henry
JC408	*MATa; hac1::URA3; ura3-1; leu2-3,-112::LEU-UPRE-lacZ; his3-11,-15; trp1-1; ade2-1; can1-100*	Cox and Walter (1996)

4.12-kb *Bam*HI-*Nru*I fragment containing the complete *HMG1* open reading frame from pJR435 (Basson *et al.*, 1988) in front of a 700-bp *Eco*RI-*Bam*HI fragment containing the *GAL1/10* promoter in pRS314. This places *HMG1* under the control of the *GAL1/10* promoter.

RNA Analyses

S1 Nuclease Protection Assay. S1 nuclease protection assays were carried out as described by Favaloro *et al.* (1980). Briefly, 15 μg of RNA were hybridized at 37°C overnight to 0.01 pmol of each probe. S1 digestion was carried out at 18°C for 2 h using 500 U/ml of S1 nuclease (Sigma). Samples were then fractionated on 8% polyacrylamide-denaturing gels and analyzed by autoradiography and by quantitation using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Northern Blots. Cells were treated and total RNA prepared as described previously (Cox *et al.*, 1993). RNA was quantitated and equal amounts were loaded on 6.7% formaldehyde and 1.5% agarose gels and run in 1× E buffer (20 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.0, 5 mM NaOAc, 0.5 mM EDTA). The RNA was transferred to Duralon-UV membranes (Stratagene,La Jolla, CA) and probed overnight at 65°C in Church hybridization buffer (0.5 M $NaPO_4$, pH 7.2, 7% SDS, 1 mM EDTA). The membranes were subsequently washed in 0.5–1× SSC and exposed. Quantitation of Northern blots was performed on a Molecular Imager System GS-363 (Bio-Rad, Richmond, CA). All Northern probes were labeled with [α-^{32}P]dCTP using the Ready-To-Go DNA labeling kit (Pharmacia, Pistcatway, NJ). The *INO1* and *ACT1* probes were generated by polymerase chain reaction of 1-kb fragments of the respective coding regions.

Western Blots and Immunoprecipitations

The anti-hemagglutinin (HA) ascites fluid was purchased (BAbCO, Richmond, CA). Denatured protein extracts were prepared by bead-beating cells directly into 10% trichloroacetic acid, 10 mM Tris (pH 8.0), 25 mM NH_4OAc, and 1 mM EDTA. Trichloroacetic acid precipitates were resuspended in 100 mM Tris (pH 11.0) and 3% SDS, and heated to 100°C for 5 min. SDS-PAGE was performed on 10–15% gradient gels, and Western blots were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) according to the instructions of the manufacturer.

For pulse labelings, wild-type cells (JC104) were grown in media either containing or lacking inositol for 6 h. Three to five OD_{600} units of cells were harvested, resuspended in the same medium at a density of 1 per ml, and incubated for 5 min with 50 μCi of [^{35}S]methionine (Amersham) per OD_{600} unit. Tm-treated cells were incubated for 15 min in medium before labeling. An amount of cell extract corresponding to an equivalent number of cells was subjected to immunoprecipitation using anti-carboxypeptidase Y (CPY) antibodies.

RESULTS

Mutant cells that cannot mount a UPR are inositol auxotrophs. Upon inositol depletion from the growth media, growth of Δ*ire1* mutant cells begins to slow at about 5 h and ceases after about 10 h (Figure 1), raising the intriguing question of how and to what purpose

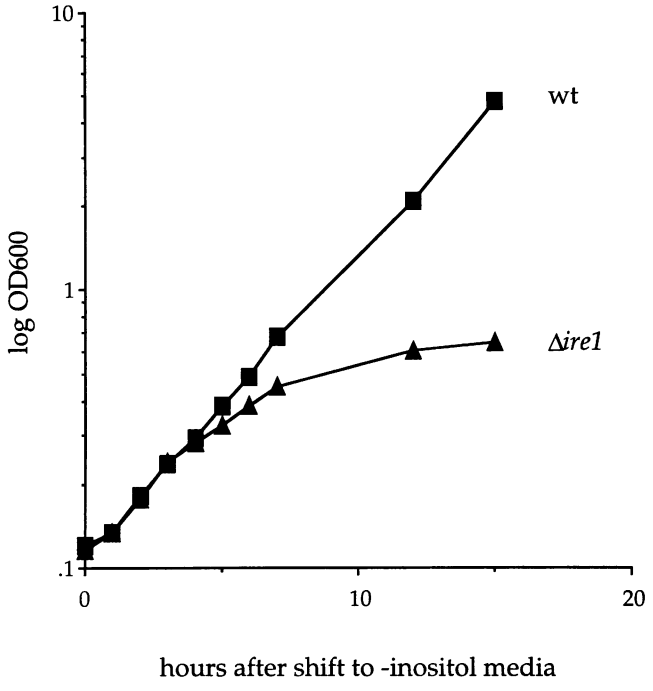

Figure 1. Growth of wild-type and ire1 mutants upon inositol starvation. Cells grown in minimal media containing inositol were collected by centrifugation, washed, and diluted into minimal media lacking inositol. Growth of wild-type (squares) and Δ*ire1* mutant strains (triangles) was assessed by optical density at 600 nm. wt, wild type.

the UPR and the inositol biosynthesis pathways are linked.

A key control point in inositol biosynthesis in yeast is the regulated expression of Ino1p, the enzyme that catalyzes synthesis of inositol from glucose. When wild-type cells are grown in the absence of inositol, the transcription of *INO1* is induced. We therefore tested whether UPR mutant cells are impaired for *INO1* induction. To address this question genetically, we expressed *INO1* from a multicopy plasmid in Δ*ire1* mutant cells. As shown in Figure 2A (lower right), expression of *INO1* allowed the Δ*ire1* cells to grow on plates lacking inositol. Similarly, when *INO1* was derepressed in Δ*ire1* mutant cells by disruption of *OPI1*, encoding a transcriptional repressor of *INO1*, the resulting Δ*ire1*Δ*opi1* double mutant also grew on media lacking inositol (Figure 2A, upper right). Taken together, these results suggest that inositol auxotrophy of Δ*ire1* mutant cells results from an impaired ability to transcribe *INO1* upon inositol starvation.

To address this possibility directly, we determined the levels of *INO1* mRNA in the strains described in Figure 2A using S1 nuclease protection of RNA harvested from cells that were grown either in the presence or absence of inositol. As expected, wild-type cells dramatically induced the transcription of *INO1* when shifted for 6 h to media lacking inositol (Figure 2B, lanes 1 and 2). Unexpected from the growth phenotypes in Figure 2A, however, we found that Δ*ire1* mutant cells also induced *INO1* transcription, albeit only to about 25% of the levels found for the wild-type strain (Figure 2B, lanes 3 and 4). Similar results were obtained for *OPI3*, which is coregulated with *INO1* during the inositol response (Figure 2B). *INO1* was expressed at higher levels in both the Δ*ire1*Δ*opi1* double mutant strain (Figure 2B, lanes 5 and 6) and the Δ*ire1* strain containing *INO1* in high copy than in the Δ*ire1* strain alone (Figure 2B, lanes 7 and 8). Thus, it appears that the level of *INO1* transcription is critical, since the reduced level found in Δ*ire1* mutant cells is insufficient to confer inositol prototrophy to the cells.

To analyze the partial induction of *INO1* transcription seen in Δ*ire1* mutants cells in more detail, we determined the time course of *INO1* induction in response to inositol depletion. As shown in Figure 3, wild-type cells induced *INO1* maximally 3 h after inositol depletion and maintained high *INO1* mRNA levels throughout the time course (Figure 3, lanes 1–5, squares). In contrast, Δ*ire1* mutants induced *INO1* transcription initially, but only reached 75% of the wild-type levels by 3 h and were then unable to sustain high levels of *INO1* transcription (Figure 3, lanes 6–10, triangles). Instead, *INO1* mRNA levels dropped to about 25% of the response observed in wild-type cells. Δ*ire1* cells transcribed *ACT1* at the later points of the time course, suggesting that the drop of *INO1* transcription was a specific effect and not caused indirectly as a result of cessation of cell growth.

From these data, we conclude that *ire1* mutants are inositol auxotrophs because they cannot maintain the high levels of *INO1* transcription required for sufficient de novo inositol biosynthesis. Furthermore, it appears that the signaling events leading to an activation of *INO1* transcription in response to depletion of inositol can be broken down into two steps: an initial *IRE1*-independent and a sustained *IRE1*-dependent induction of *INO1* transcription.

Since *IRE1* is required for growth in inositol-depleted media, we reasoned that the Ire1p kinase may become activated under these conditions, thus inducing the UPR. Indeed, S1 nuclease analysis showed that *KAR2* transcription was induced fourfold after inositol depletion (Figure 2B, lanes 1 and 2). As expected, this induction was strictly *IRE1* dependent; no induction of *KAR2* transcription in response to inositol depletion was observed in Δ*ire1* mutant cells (Figure 2B, lanes 3–8). Thus, we conclude that inositol depletion activates the UPR.

Since inositol is a sugar and a precursor in carbohydrate synthesis as well as lipid synthesis, it is possible that depletion of inositol indirectly activates the UPR by causing protein glycosylation defects in the ER. To rule out this possibility, we monitored the extent of glycosylation of CPY in wild-type cells starved for inositol. As shown in Figure 3C, most of the newly synthesized CPY is highly glycosylated in cells grown in the presence of inositol and in cells shifted to inositol depleted media for 6 h (Figure 3C, lanes 1 and 2). Therefore, activation of Ire1p in response to inositol depletion cannot be attributed to a general defect in protein glycosylation and is likely to be a direct response to cellular inositol levels.

It therefore became important to test the reciprocal scenario, i.e., to test whether the accumulation of unfolded proteins in the ER (which induces the UPR) also activates *INO1* transcription. As shown in Figure 4, Tm treatment (which induces unfolded proteins in the ER by impairing protein glycosylation) induced *INO1* transcription significantly (Figure 4, lanes 1–5). *INO1* was induced approximately 300-fold at the 9-h time point. As expected, *KAR2* transcription was also induced. The induction of both *INO1* and *KAR2* required Ire1p, as demonstrated by the fact that a Δ*ire1* mutant was unable to up-regulate transcription of either gene (Figure 4, lanes 6–10). Thus, we conclude that accumulation of unfolded proteins in the ER activates *INO1* transcription. Ire1p therefore participates in two regulatory pathways: the UPR and the transcriptional regulation of *INO1* in response to inositol starvation.

Since Hac1p is a transcription factor required for the UPR and is the component of the cascade most distal to the unfolded protein signal, we next tested

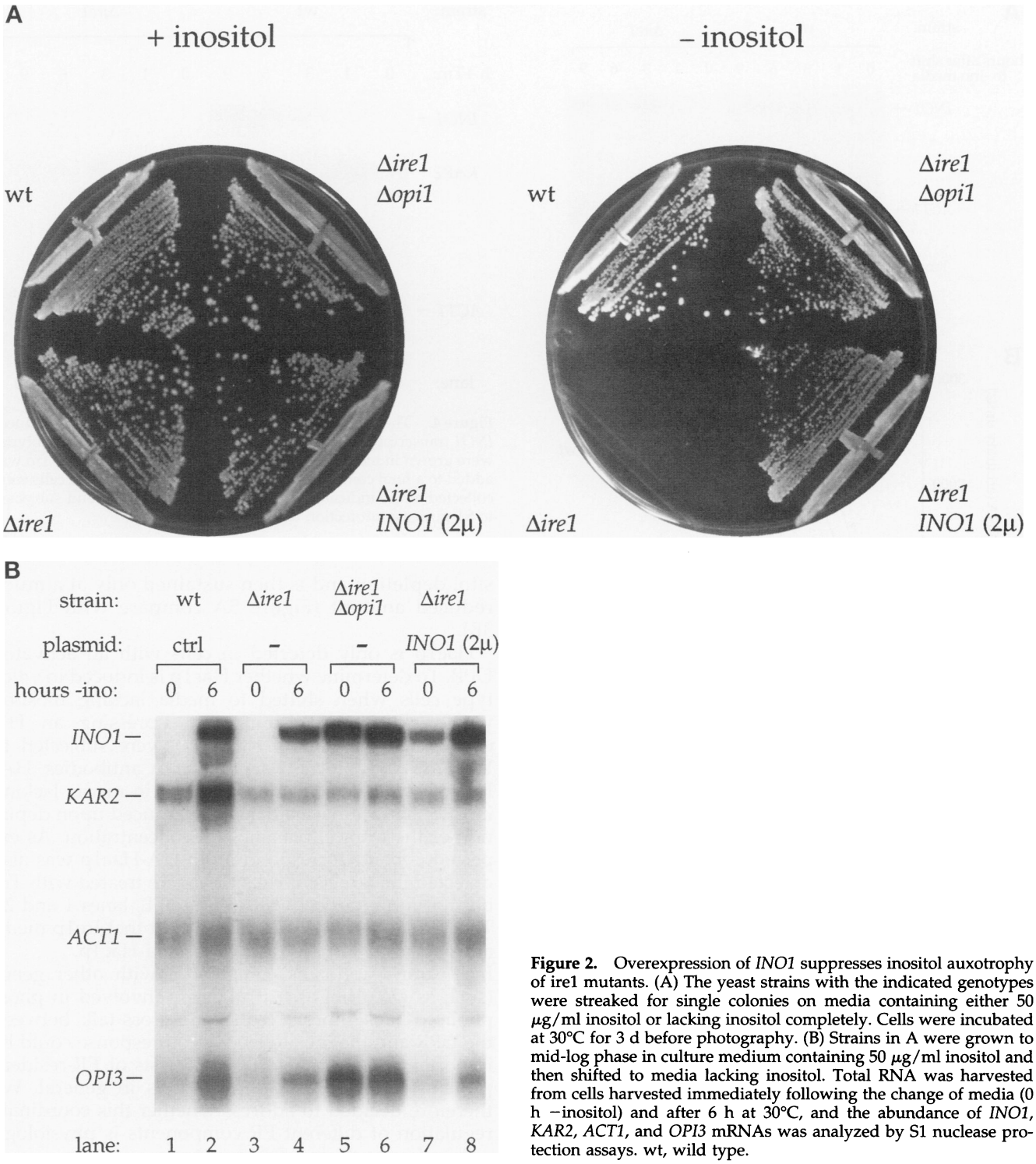

Figure 2. Overexpression of *INO1* suppresses inositol auxotrophy of ire1 mutants. (A) The yeast strains with the indicated genotypes were streaked for single colonies on media containing either 50 μg/ml inositol or lacking inositol completely. Cells were incubated at 30°C for 3 d before photography. (B) Strains in A were grown to mid-log phase in culture medium containing 50 μg/ml inositol and then shifted to media lacking inositol. Total RNA was harvested from cells harvested immediately following the change of media (0 h −inositol) and after 6 h at 30°C, and the abundance of *INO1*, *KAR2*, *ACT1*, and *OPI3* mRNAs was analyzed by S1 nuclease protection assays. wt, wild type.

whether mutations in *HAC1* also affect the inositol-signaling pathway. Two lines of evidence suggest that this is indeed the case. First, Δ*hac1* mutants are inositol auxotrophs (Sidrauski *et al.*, 1996). Second, the induction profile of *INO1* in response to depletion of inositol in the Δ*hac1* mutant is indistinguishable from that seen in Δ*ire1* mutants: the *INO1* mRNA level peaks in Δ*hac1* mutants 3 h after ino-

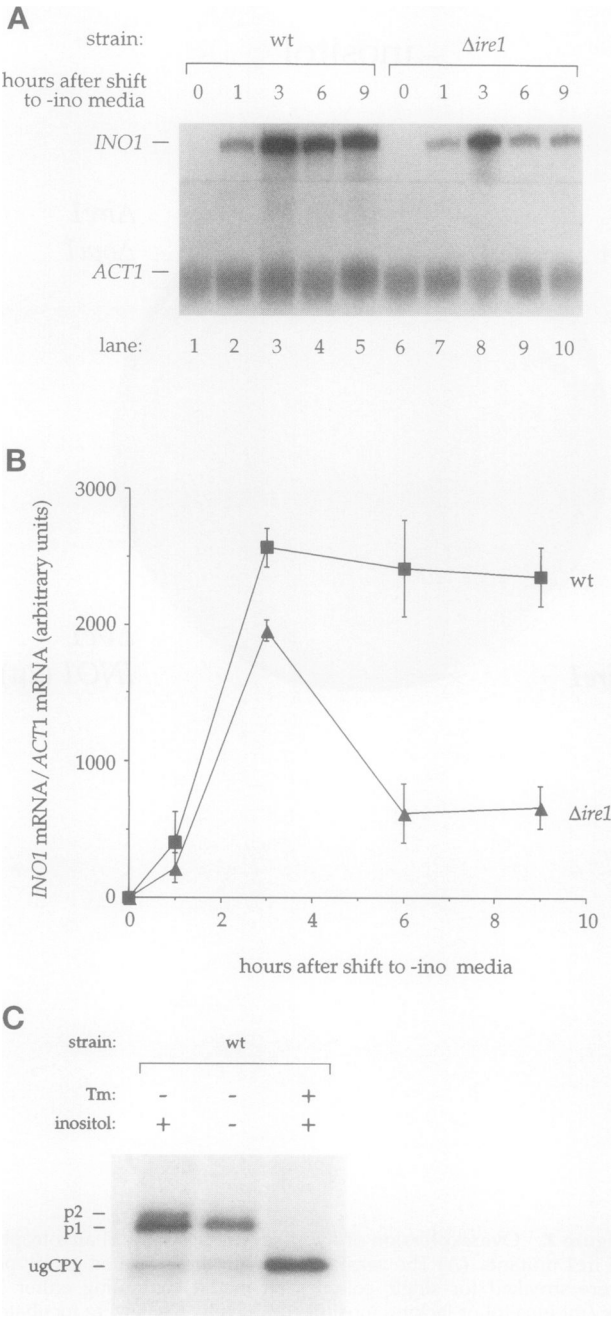

Figure 3. *ire1* mutants are unable to sustain *INO1* transcriptional induction. (A) Wild-type and Δ*ire1* mutant strains were grown in the presence of 50 μg/ml inositol and then shifted to media lacking inositol. S1 protection analysis was performed on RNA isolated from cells harvested at different time points after the media shift and probed for *INO1* and *ACT1* mRNAs. (B) The data from A and a repeat experiment were quantitated and normalized to *ACT1* mRNA levels. (C) Wild-type cells were pulse labeled for 5 min with [^{35}S]methionine after growth for 6 h in media either containing (+INO) or lacking (−INO) inositol. Cell extracts were prepared and CPY immunoprecipitates were analyzed by SDS-PAGE. CPY from Tm-treated cells (+Tm) serves as a marker for the unglycosylated form of the protein (μgCPY), and the ER glycosylated (p1) and Golgi glycosylated (p2) forms are indicated. wt, wild type.

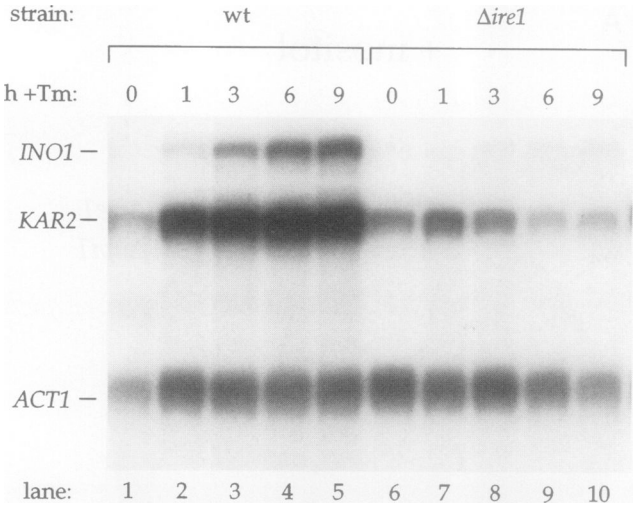

Figure 4. The accumulation of unfolded proteins in the ER induces *INO1* transcription. Cultures of strains with the indicated genotypes were grown in media containing 50 μg/ml inositol at 30°C. Tm was added to a final concentration of 1 μg/ml, and aliquots of cells were collected at the indicated times. RNA was harvested and subjected to S1 nuclease protection analysis. wt, wild type.

sitol depletion and is then sustained only at a much reduced amount (Figure 5A compare with Figure 3B).

Hac1p is only detected in cells with an activated UPR. To determine whether Hac1p is induced in wild-type cells when shifted to media lacking inositol, whole-cell extracts from cells expressing an HA epitope-tagged version of Hac1p were subjected to Western blot analysis using anti-HA antibodies. HA-Hac1p was detected in cells grown in media lacking inositol, indicating that Hac1p is induced upon depletion of the intracellular inositol concentration. As expected, a band corresponding to HA-Hac1p was also detected in extracts of cells that were treated with Tm but not in uninduced cells (Figure 5B, lanes 1 and 2). Taken together, these results suggest that Ire1p mediates its effects on *INO1* induction via Hac1p.

INO1 transcription is coregulated with other genes (such as *OPI3*, Figure 2B) that are involved in phospholipid biosynthesis. Thus, the cross-talk between the UPR and the inositol starvation response could be important to coordinate the synthesis of ER-resident proteins with membrane biogenesis in general. We therefore decided to address whether this coordinate regulation of different ER components is physiologically relevant during ER biogenesis.

When one of a number of ER membrane proteins, such as HMG-CoA reductase, is overexpressed, yeast cells increase the amount of ER to produce a specialized membrane system called karmellae (Wright *et al.*, 1988). Karmellae are stacks of layered membranes partially surrounding the nucleus that are continuous

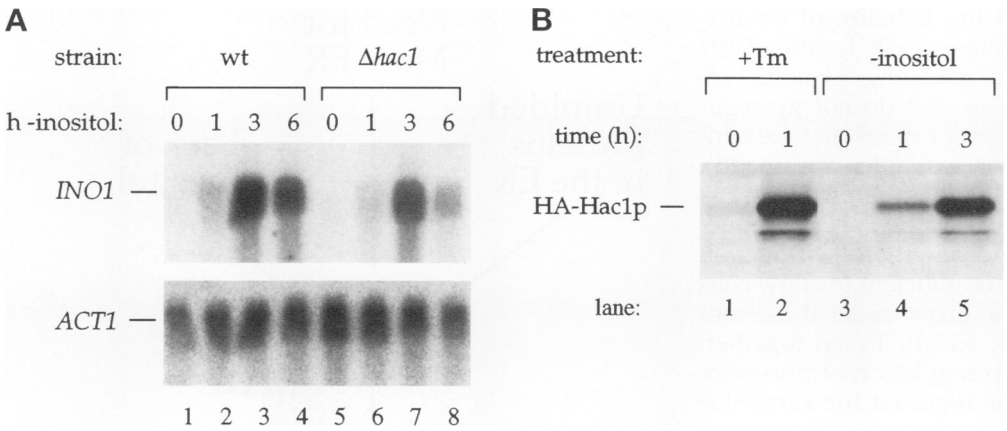

Figure 5. Hac1p is required for inositol signaling. (A) Δ*hac1* mutant cells were grown to mid-log phase in the presence of 50 μg/ml inositol and then shifted to media lacking inositol. Total RNA harvested from cells taken at different time points after the media change were subjected to Northern blot analysis using an *INO1* probe. The blot was stripped and reprobed for *ACT1* mRNA removal. (B) Δ*hac1* mutants harboring the HA-tagged version of the *HAC1* gene were grown either in the absence or presence of Tm (lanes 1 and 2) or shifted to media lacking inositol (lanes 3- 5). Cell extracts were probed by Western blot analysis using α-HA antibodies. wt, wild type.

with the nuclear envelope and enclose ER-resident proteins. Therefore, the ability to induce karmellae formation allowed us to specifically regulate ER biogenesis.

To determine whether the *IRE1*-dependent regulation of synthesis of ER components is required for karmellae formation, we overexpressed the gene encoding HMG-CoA reductase, *HMG1*, from an inducible *GAL* promoter in both wild-type and Δ*ire1* mutant cells. As shown in Figure 6, overproduction of HMG-CoA reductase in Δ*ire1* mutants (Figure 6; Δ*ire1*, +Gal) but not in the wild-type parental strain (Figure 6; wt, +Gal) was lethal. Since Ire1p is required for both the UPR- and inositol-signaling pathways, Δ*ire1* mutant cells could be dying due to an inability to induce ER-resident proteins, phospholipid biosynthetic enzymes, or both. To distinguish between these possibilities, we first asked whether deletion of *OPI1* in the

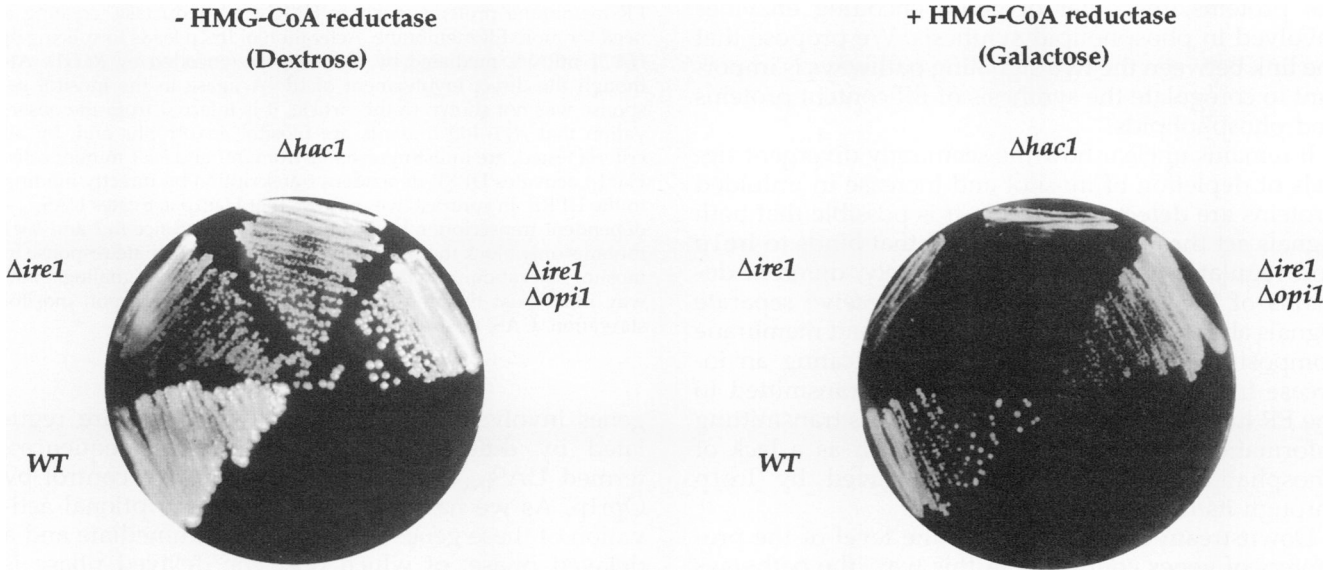

Figure 6. Overexpression of HMG-CoA reductase impairs growth of strains that are blocked in the inositol response. Yeast strains of the indicated genotypes containing HMG-CoA reductase under the control of the GAL1/10 promoter (pRC1) were plated for single colonies on minimal plates containing either dextrose or galactose as the sole carbon source. Cells were incubated at 30°C for 3 d before photography. WT, wild type.

Δ*ire1* strain would suppress the lethality of overexpression of HMG-CoA reductase. Cells lacking *OPI1* constitutively express genes encoding proteins required for membrane synthesis, but do not up-regulate genes encoding ER-resident proteins that are controlled by the UPR (see, for example, Figure 2B). Indeed, the Δ*ire1* Δ*opi1* double mutant grew almost as well as the wild-type strain when HMG-CoA reductase was overexpressed, suggesting that the induction of phospholipid biosynthesis is sufficient to allow cells that cannot induce the UPR to grow under these conditions (Figure 6; Δ*ire1*Δ*opi1*, +Gal). Taken together, these data suggest that an up-regulation of phospholipid biosynthesis enzymes is required for karmellae synthesis.

DISCUSSION

We have shown that the pathways regulating synthesis of ER lumenal and phospholipid biosynthetic enzymes are intimately linked. In particular, we have demonstrated that two components, Ire1p and Hac1p, function as common signal mediators in both pathways. The coordinate regulation requires the activity of Ire1p, an ER membrane receptor kinase. Ire1p therefore becomes activated in response to different input signals: one resulting from inositol depletion, another from an accumulation of unfolded protein in the ER lumen, and a third upon overproduction of an ER membrane protein (Figure 7). Ire1p activation leads to the stable expression of Hac1p, which in turn produces two different output signals: one leading to an increase in transcription of genes encoding ER lumenal proteins, the other of genes encoding enzymes involved in phospholipid synthesis. We propose that the link between the two signaling pathways is important to coregulate the synthesis of ER content proteins and phospholipids.

It remains unclear how the seemingly divergent signals of depletion of inositol and increase in unfolded proteins are detected by Ire1p. It is possible that both signals act through a single ligand that binds to Ire1p and regulates its activity. Alternatively, different domains of the Ire1p molecule might receive separate signals about the state of the ER lumen and membrane composition. For example, signals indicating an increase in unfolded proteins might be transmitted to the ER lumenal part of Ire1p, and signals transmitting information about the membrane, such as a lack of phosphatidylinositol, might be received by Ire1p through its transmembrane domain.

Downstream of Hac1p, i.e., at the level of the promoters of genes controlled in this way, the pathways diverge. Transcriptional induction of genes encoding ER lumenal proteins requires the presence of a UPRE in the promoter to which Hac1p binds directly and acts as a positive transcription factor. In contrast, the genes involved in phospholipid synthesis are regulated by different upstream activating sequences, termed UAS_{INO}, that are under negative control by Opi1p. As we have shown here, transcriptional activation of these genes shows both an immediate and a delayed phase, of which only the delayed phase is dependent on Ire1p and Hac1p. Thus, in the absence of either component, an immediate response to inositol starvation is still transmitted to the transcriptional apparatus via an unknown route but cannot be sus-

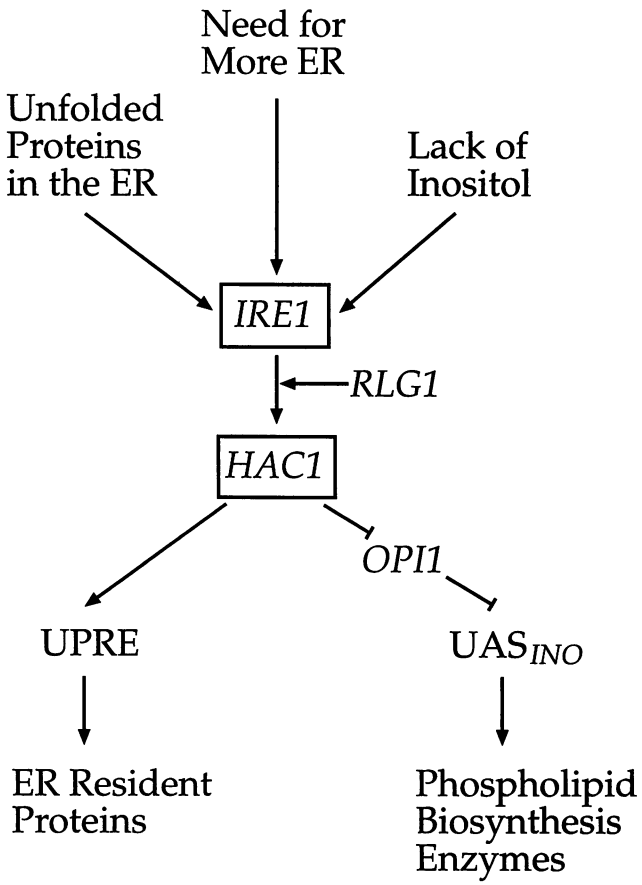

Figure 7. Ire1p and Hac1p coordinate the UPR and inositol starvation response. The UPR and inositol response converge on both Ire1p and Hac1p. Ire1p can also be activated upon overexpression of ER membrane proteins, such as HMG-CoA reductase, creating a need for more ER membrane. Activation of Ire1p leads to splicing of *HAC1* mRNA, mediated by tRNA ligase (encoded by *RLG1*). Although the direct involvement of tRNA ligase in the inositol response was not shown in this article, it is inferred from the observation that *rlg1–100* mutants are inositol auxotrophs and, by all criteria tested, are indistinguishable from *ire1* and *hac1* mutant cells. Hac1p activates UPRE-dependent transcription by directly binding to the UPRE. In contrast, we propose that Hac1p activates UAS_{INO}-dependent transcription by inactivating Opi1p. Since *ire1* and *hac1* mutants only block the sustained but not the immediate response to inositol starvation, an additional, uncharacterized signaling pathway must exist that induces the immediate phase upon inositol starvation. UAS, upstream-activating sequence.

tained at a sufficiently high level to confer inositol prototrophy. It is likely that this immediate response inactivates Opi1p temporarily, possibly by phosphorylation, whereas the Ire1p- and Hac1p-dependent response is required to sustain Opi1p's inactivation.

How might Hac1p direct transcription from the UAS_{INO}? It is possible that Hac1p 1) binds directly to the UAS_{INO}, or adjacent sequences to activate transcription of downstream genes; 2) that Hac1p antagonizes Opi1p repressor activity; or 3) that Hac1p acts indirectly by inducing transcription of another gene which then antagonizes Opi1p repressor activity. We consider the first possibility unlikely because in Δ*opi1* mutants, genes controlled by the UAS_{INO} are fully and constituitively transcribed even in the absence of Hac1p. Furthermore, a previously identified sequence resembling a UPRE in the *INO1* promoter did not compete with the *KAR2* UPRE for Hac1p binding when tested in gel shift experiments (Cox and Walter, unpublished observation), thus abolishing the need to invoke direct binding of Hac1p to UAS_{INO}-controlled promoters in our models.

We favor the second possibility suggested above, i.e., that Hac1p influences UAS_{INO}-containing genes by antagonizing Opi1p repressor activity. Because both Hac1p and Opi1p both contain putative leucine zipper domains, one intriguing possibility would be that these proteins interact by forming an inactive or unstable heterodimer. According to this model, expression of Hac1p in excess of the amount needed to activate UPRE-controlled promoters may squelch Opi1p by preventing it from binding to the UAS_{INO}, activating transcription of inositol-regulated genes. Intriguingly, it has been shown previously that when inositol levels drop, Opi1p becomes ubiquitinated and targeted for degradation by the proteasome (McGraw, personal communication). We have demonstrated recently that Hac1p is a short-lived protein, which is also targeted to degradation by the same ubiquitin conjugating enzymes, Ubc4p and Ubc5p, as is Opi1p (Cox and Walter, 1996; in wild-type cells, the half-life of the induced form $Hac1p^i$ is about 4 min). Heterodimerization might therefore promote the degradation of Opi1p. This model predicts that mutations in Hac1p that abolish only DNA-binding activity would prevent the activation of UPRE-controlled promoters yet retain its ability to interact with Opi1p and hence be able to derepress UAS_{INO}-controlled promoters. Indeed, we have been able to isolate mutations in *HAC1* that selectively impair UPRE-dependent transcription, but still allow induction of UAS_{INO}-controlled promoters (Chapman and Walter, unpublished observations). With these tools, we can now test whether Hac1p directly interacts with Opi1p and, if so, how this interaction affects UAS_{INO}-dependent transcription.

Most important, we have demonstrated that the control of UAS_{INO}-regulated genes via the Ire1p/Hac1p-dependent signaling pathway is important under certain physiological conditions. Conditions which induce karmellae, a specialized form of ER, are lethal for cells lacking Ire1p or Hac1p if inositol is limiting. However, *ire1* mutant cells are viable and able to make karmellae in the absence of Ire1p if either 1) sufficient inositol is provided in the growth medium or 2) the *OPI1* gene is also mutated, resulting in derepressed transcription of its target genes. These data indicate that the inability to make more membrane, rather than saturation of the protein folding machinery, causes lethality in *ire1* mutant cells during karmellae induction. We propose that when phospholipid biosynthesis becomes limiting in *ire1* mutant cells, high levels of HMG-CoA reductase cannot be properly accommodated in the membrane, leading to aberrant ER function that blocks cell growth. According to this view, the UPR pathway is essential to maintain a proper balance between the biosynthesis of protein and lipid components of the ER. This is an appealing model as the coupling of the transcriptional networks regulating both the lumenal and lipid components of the ER would allow the cell to precisely coordinate the amount of the organelle.

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