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Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus

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The transmembrane kinase Ire1p is required for activation of the unfolded protein response (UPR), the increase in transcription of genes encoding endoplasmic reticulum (ER) resident proteins that occurs in response to the accumulation of unfolded proteins in the ER. Ire1p spans the ER membrane (or the nuclear membrane with which the ER is continuous), with its kinase domain localized in the cytoplasm or in the nucleus. Consistent with this arrangement, it has been proposed that Ire1p senses the accumulation of unfolded proteins in the ER and transmits the signal across the membrane toward the transcription machinery, possibly by phosphorylating downstream components of the UPR pathway. Molecular genetic and biochemical studies described here suggest that, as in the case of growth factor receptors of higher eukaryotic cells, Ire1p oligomerizes in response to the accumulation of unfolded proteins in the ER and is phosphorylated *in trans* by other Ire1p molecules as a result of oligomerization. In addition to its kinase domain, a C-terminal tail domain of Ire1p is required for induction of the UPR. The role of the tail is probably to bind other proteins that transmit the unfolded protein signal to the nucleus.
Keywords: BiP/PDI/*S.cerevisiae*/transmembrane receptor/unfolded protein response

Introduction

When unfolded proteins accumulate in the endoplasmic reticulum (ER), the unfolded protein response (UPR) is induced, which results in the increased transcription of genes encoding ER resident proteins. Thus, the unfolded protein signal that originates in the ER follows a path to the nucleus. The proteins whose expression is increased as a result of the response are chaperones and enzymes such as BiP and protein disulfide isomerase (PDI) that function to assist in the correct folding of secreted proteins that pass through the ER.

The UPR pathway is conserved through evolution in organisms as divergent as mammals and yeast. In *Saccharomyces cerevisiae*, transcription of the genes *KAR2* (encoding BiP), *PDI1* (encoding PDI), *EUG1* (encoding a PDI-like protein) and *FKB2* (encoding peptidyl-prolyl *cis–trans* isomerase) is up-regulated as a result of the UPR (for recent reviews, see Sweet, 1993; McMillan *et al.*, 1994; Shamu *et al.*, 1994). The response can be induced experimentally in yeast by preventing glycosylation either by treating cells with the drug tunicamycin or by mutating *SEC53*, the gene encoding phosphomannomutase.

Two components of the *S.cerevisiae* UPR pathway have been identified. The unfolded protein response element (UPRE) is a 22 bp upstream activating sequence that is necessary and sufficient to activate transcription of a linked promoter in response to the accumulation of unfolded proteins in the ER (Mori *et al.*, 1992; Kohno *et al.*, 1993). The second known component of the pathway is the transmembrane kinase Ire1p, encoded by the non-essential gene *IRE1*. *ire1* cells do not increase transcription of *KAR2*, *PDI1* or *EUG1* in response to the accumulation of unfolded proteins in the ER (Cox *et al.*, 1993; Mori *et al.*, 1993). *IRE1* was identified originally because it is required for inositol prototrophy. *ire1* mutants do not grow in the absence of inositol (Nikawa and Yamashita, 1992). Models to explain the link between inositol metabolism and the UPR have been proposed (Cox *et al.*, 1993; Mori *et al.*, 1993), but the relationship between the two pathways is still not fully understood.

To date, Ire1p is the only transmembrane kinase to have been identified in yeast. It is 1115 amino acids long and has a signal sequence at the N-terminus, a single transmembrane domain located approximately in the middle of the protein and a kinase domain near the C-terminus. The N-terminal half of Ire1p is core-glycosylated and lies in a membrane-bounded compartment (Mori *et al.*, 1993). Thus, it is likely that the Ire1p N-terminal domain lies in the lumen of the ER, where it presumably detects the accumulation of unfolded proteins, and that the kinase domain lies in the cytoplasm (or nucleus), where it presumably phosphorylates downstream components of the UPR pathway. Point mutations affecting residues of Ire1p conserved among all kinases prevent activation of the UPR (Mori *et al.*, 1993), suggesting that the kinase activity of Ire1p is required for the UPR.

Sequence comparisons with other kinases suggest that Ire1p is a serine/threonine kinase (Hanks *et al.*, 1988). Thus, Ire1p is a member of the class of transmembrane serine/threonine kinases that includes the transforming growth factor (TGF)-β type II receptor. However, the Ire1p kinase domain has no close relatives among known kinases (Hanks and Hunter, 1995).

After its kinase domain, Ire1p has a C-terminal 'tail' domain that is ~133 amino acids long. Many receptor tyrosine kinases also have protein tails that lie C-terminal to their kinase domains. In most cases, these tails interact with downstream components of the signaling pathway (Heldin, 1995 and references therein). The function of the Ire1p tail is unknown, and sequence comparisons with the kinases most related to Ire1p provide few clues. For example, only some members of the TGF-β family of transmembrane receptors have tails after their kinase

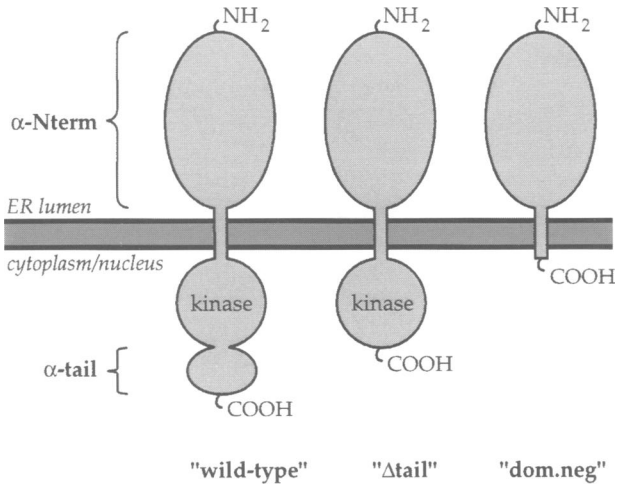


Fig. 1. Specificity of antibodies and schematic depiction of Ire1p truncation mutants. The epitopes used to produce the α-Ire1p antibodies α-Nterm and α-tail, and the Ire1p mutants 'tailless' (Δtail; truncated after amino acid 982) and dominant negative (dom. neg; truncated after amino acid 674) are diagrammed.

domains (Derynck, 1994; Massague *et al.*, 1994). At least in the case of the TGF-β type II receptor, deletion of the tail does not diminish signaling activity (Wrana *et al.*, 1994). Thus, we cannot argue the function of the Ire1p tail by analogy.

Understanding the mechanism by which the Ire1p kinase is activated is a first step towards understanding how the accumulation of unfolded proteins in the ER is sensed by Ire1p and then transmitted to other proteins in the cytoplasm or nucleus. Most receptor transmembrane kinases are regulated by phosphorylation and oligomerization (Heldin, 1995). Using a combination of molecular genetic and biochemical approaches, we have examined the oligomerization and phosphorylation states of Ire1p. Our data support the model that Ire1p oligomerizes as unfolded proteins accumulate in the ER, that it is phosphorylated *in trans* by other Ire1p molecules as a result of oligomerization, and that both oligomerization and phosphorylation of Ire1p are required for activation of the UPR.

Results

As a first step in characterizing the role of the Ire1p kinase in the UPR, we raised polyclonal antibodies directed against two non-overlapping domains of Ire1p: 'α-Nterm', directed against amino acids 20–521, and 'α-tail', directed against amino acids 976–1115 (Figure 1). As shown in Figure 2A, both antisera immunoprecipitated ^{35}S-labeled Ire1p from extracts of cells expressing the protein at wild-type levels (lanes 1 and 7), but not from extracts of cells in which *IRE1* was deleted (Δ*ire1*; lanes 5 and 11). The antisera also recognized Ire1p on Western blots of extracts made from cells overexpressing the protein (not shown); however, no specific bands could be detected on Western blots of extracts from wild-type yeast cells, in agreement with the previous suggestion that Ire1p is not very abundant (Mori *et al.*, 1993). As expected, the α-Nterm antibodies also immunoprecipitated a truncation mutant of Ire1p that is deleted for the C-terminal 133 amino acids ('Δtail', Figure 1), whereas the α-tail antibodies did not immunoprecipitate this protein (Figure 2A, compare lanes 3 and 9).

It has been suggested that Ire1p might be proteolyzed upon induction of the UPR (Mori *et al.*, 1993). To test this possibility directly, we immunoprecipitated Ire1p from extracts of wild-type cells that had been labeled with [^{35}S]methionine and grown in the presence or absence of tunicamycin, an inducer of the UPR. As shown in Figure 2A, the levels of Ire1p did not change significantly with the induction of the UPR, and we did not detect proteolytic fragments of Ire1p corresponding to either the N- or C-terminal half of the protein in extracts of cells treated with tunicamycin (Figure 2A, lanes 2 and 8). Rather, we observed reproducibly that the mobility of Ire1p decreased slightly after treatment with tunicamycin (this is best seen by comparing the distance of the Ire1p band with the minor contaminant just below it in lanes 7 and 8 in Figure 2A). Thus, we consider it unlikely that the UPR is mediated by proteolysis of Ire1p. The slight mobility shift of Ire1p suggested, however, that other post-translational modifications of the kinase occur.

The C-terminal tail of Ire1p is required for the unfolded protein response

To investigate the function of the Ire1p tail, we constructed a tailless version of Ire1p (Figure 1) in which the coding sequence ends shortly after the kinase domain as defined by sequence alignments (Hanks and Hunter, 1995). When *ire1-Δtail* was introduced into yeast cells, replacing wild-type *IRE1* as the only copy of the gene, cells did not induce the UPR. This was determined by color assays on X-Gal–tunicamycin indicator plates, which monitor UPRE-dependent transcription of a *lacZ* reporter gene (Cox *et al.*, 1993) and by S1 nuclease protection assays to quantitate transcription of *KAR2* and *PDI1* mRNAs (Figure 2B and C). Cells bearing *ire1-Δtail* are also inositol auxotrophs (data not shown). Thus, their phenotype is identical to *ire1* mutants bearing complete deletions of the Ire1p coding sequence. Because Ire1p-Δtail is expressed as well as full-length Ire1p (Figure 2A, lanes 1–4), there are two possible explanations for this result. First, the C-terminal tail might be required for the activity of the Ire1p kinase domain itself, i.e. the *ire1-Δtail* mutant cannot carry out the UPR simply because its kinase is non-functional. Second—and more interesting—the kinase might be active in the *ire1-Δtail* mutant, suggesting that the tail is required for propagation of the unfolded protein signal. According to this latter possibility, Ire1p-Δtail might be unable to interact with downstream components of the signaling pathway.

Intragenic complementation of ire1 mutant alleles

To assess whether the kinase domain in Ire1p-Δtail is active, we asked whether the mutant protein would complement other Ire1p mutants in which the kinase activity has been selectively impaired. If successful, such intragenic complementation would also provide strong evidence for dimerization or oligomerization of Ire1p during the UPR. To this end, we used two kinase mutants, Ire1p-K702A and Ire1p-K702R (previously described by Mori *et al.*, 1993), each bearing a single amino acid substitution in the catalytic lysine residue that is conserved among all kinases and positioned in the active site (Hanks and Hunter, 1995). Cells expressing only Ire1p-K702A display an *ire1* null phenotype: they are severely compromised in

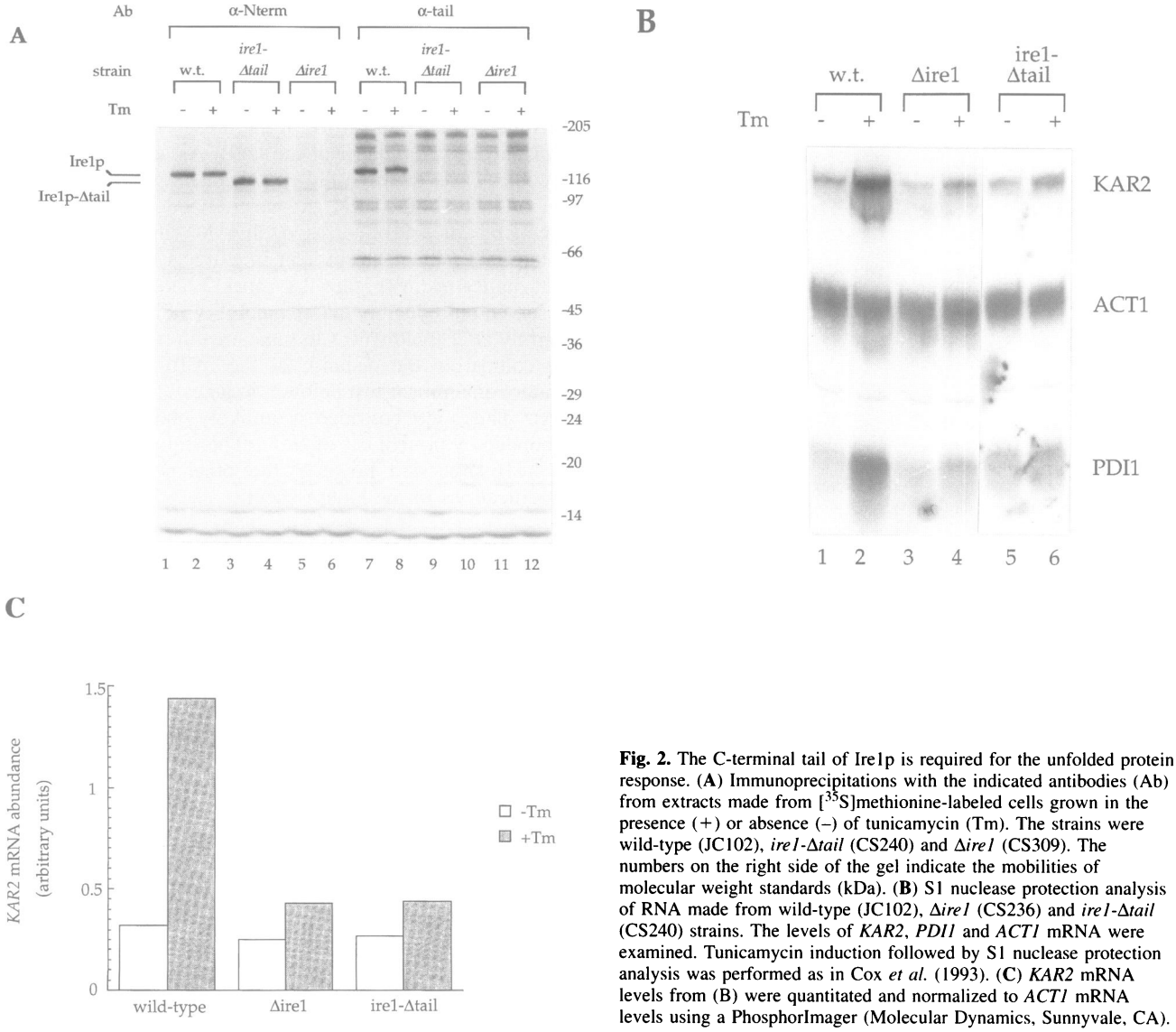


Fig. 2. The C-terminal tail of Ire1p is required for the unfolded protein response. (**A**) Immunoprecipitations with the indicated antibodies (Ab) from extracts made from [^{35}S]methionine-labeled cells grown in the presence (+) or absence (–) of tunicamycin (Tm). The strains were wild-type (JC102), *ire1-Δtail* (CS240) and *Δire1* (CS309). The numbers on the right side of the gel indicate the mobilities of molecular weight standards (kDa). (**B**) S1 nuclease protection analysis of RNA made from wild-type (JC102), *Δire1* (CS236) and *ire1-Δtail* (CS240) strains. The levels of *KAR2*, *PDI1* and *ACT1* mRNA were examined. Tunicamycin induction followed by S1 nuclease protection analysis was performed as in Cox *et al.* (1993). (**C**) *KAR2* mRNA levels from (B) were quantitated and normalized to *ACT1* mRNA levels using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

their ability to carry out the UPR and are inositol auxotrophs (Figure 3A and B). In contrast, cells expressing only Ire1p-K702R carry out a diminished UPR that is ~25% of the wild-type response (Figure 3A and B). Consistent with this finding, the K702R mutants can grow in the absence of inositol, but at a slower rate than wild-type cells. Immunoprecipitation experiments confirmed that both Ire1p-K702A and Ire1p-K702R were present in cells at the same level as Ire1p in wild-type cells (data not shown). Assuming that the UPR in these mutants reflects the activity of the Ire1p kinase in each, these data suggest that Ire1p-K702A has an inactive kinase domain whereas that of Ire1p-K702R is partially active.

To determine whether these mutant alleles could complement Ire1p-Δtail, *ire1-Δtail* cells were transformed with plasmids encoding a full-length version of either wild-type Ire1p, Ire1p-K702A or Ire1p-K702R. The ability of the transformants to carry out the UPR was assayed by S1 nuclease protection (Figure 3C and D). Interestingly, the UPR in cells carrying both Ire1p-K702R and Ire1p-Δtail was much greater than the response in cells with only Ire1p-K702R (Figure 3A and B; Figure 4A, second and eighth columns) and nearly the same as the UPR in cells in which wild-type Ire1p and Ire1p-Δtail were combined (Figure 3C, lanes 2 and 6, and Figure 3D). Cells bearing both Ire1p-K702R and Ire1p-Δtail reproducibly induced *KAR2* transcription in response to tunicamycin to levels that were >80% of the wild-type response while, as mentioned above, cells bearing only Ire1p-K702R induced *KAR2* transcription to levels that were ~25% of wild-type. Complementation between Ire1p-K702R and Ire1p-Δtail was also evident in the growth phenotypes of the cells on media lacking inositol: cells expressing both Ire1p-K702R and Ire1p-Δtail grow as well as wild-type cells (data not shown). In contrast, expression of Ire1p-K702A had no effect on the *ire1-Δtail* phenotype (Figure 3C, lanes 4 and 8, and Figure 3D), and cells expressing both Ire1p-K702A and Ire1p-Δtail did not grow on media lacking inositol. Immunoprecipitation experiments demonstrated that these phenotypes are not due to differences in expression levels of the Ire1p alleles. Wild-type Ire1p, Ire1p-K702R and Ire1p-K702A are expressed at the same level in the *Δire1* (CS309) and in the *ire1-Δtail* (CS240) strain backgrounds (data not shown).

We next asked whether Ire1p-Δtail must contain a wild-type kinase domain to complement Ire1p-K702R.

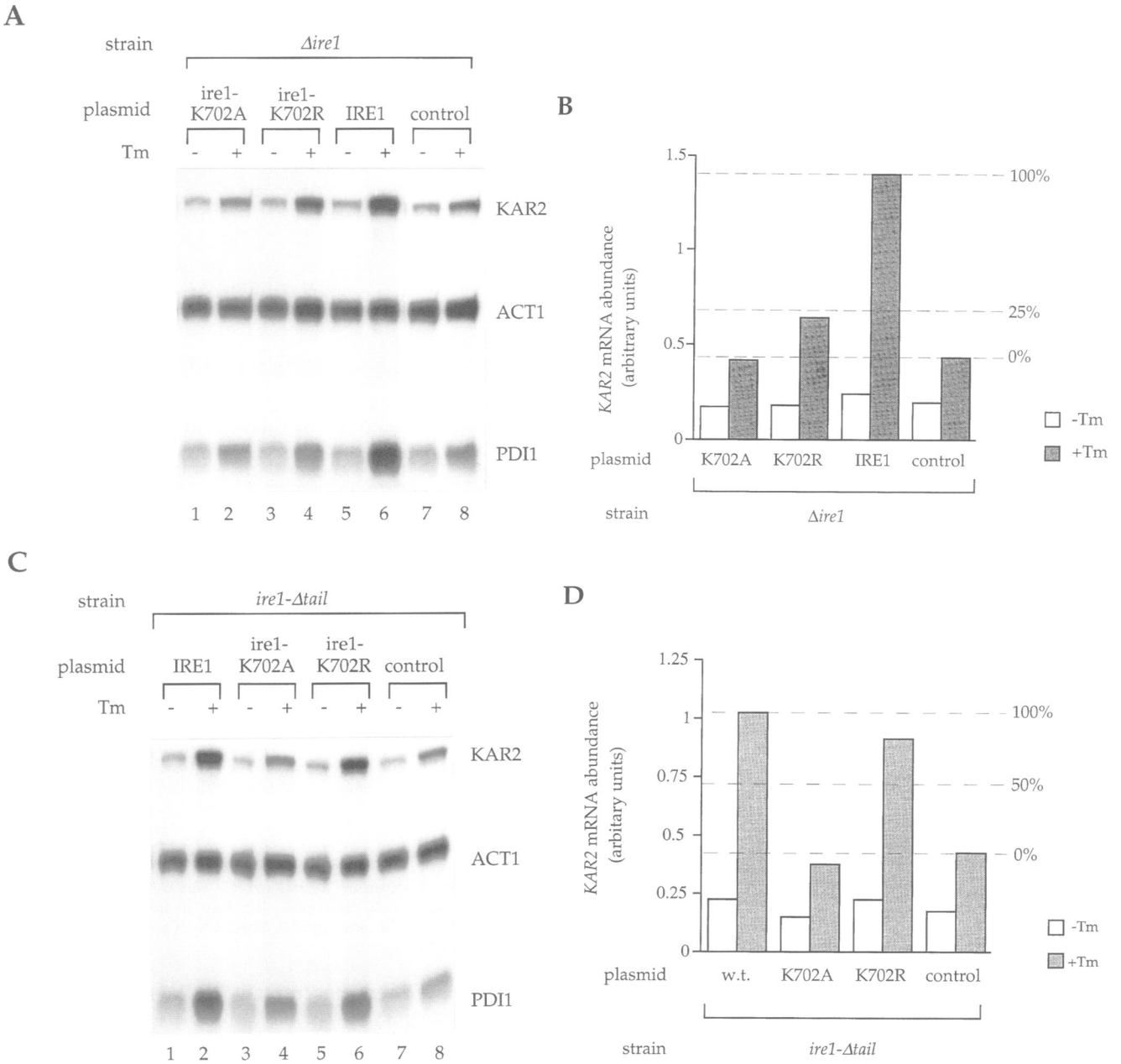
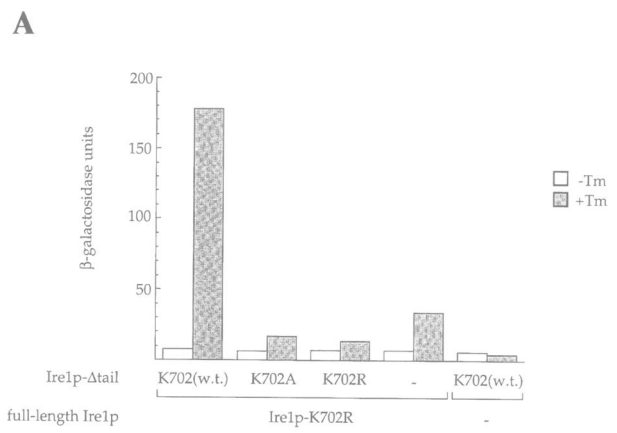
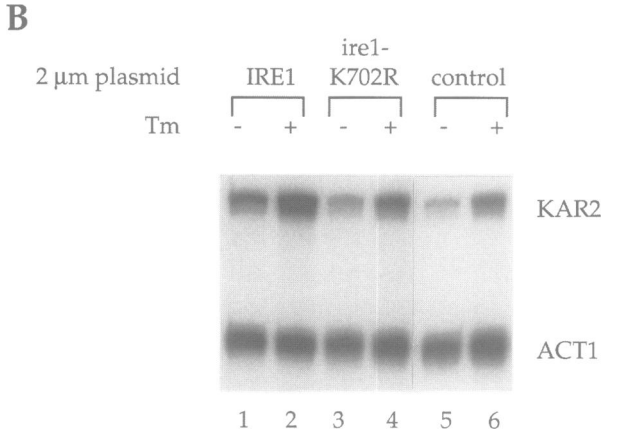


Fig. 3. Intragenic complementation between some *IRE1* alleles. Tunicamycin induction followed by S1 nuclease protection analysis was performed as described in Cox *et al.* (1993) to quantitate the unfolded protein response in each strain. The experiments as shown were carried out twice and confirmed additionally by visual color assay on tunicamycin/X-Gal indicator plates (Cox *et al.*, 1993). Results from typical experiments are presented. (**A**) S1 nuclease protection analysis of RNA made from strain CS309 (which is deleted for the Ire1p coding sequence) bearing a URA$^+$CEN/ARS plasmid with either *ire1*-K702A (plasmid pCS176), *ire1*-702R (pCS177), wild-type *IRE1* (pCS175) or no *IRE1* (control; YCplac33; Gietz and Sugino, 1988). (**B**) *KAR2* mRNA levels from (A) were quantitated and normalized to *ACT1* mRNA levels. (**C**) S1 nuclease protection analysis of RNA isolated from strain CS240 (which carries *ire1-Δtail*) bearing the CEN/ARS plasmids with various *IRE1* alleles described in (A). (**D**) *KAR2* mRNA levels from (C) were quantitated and normalized to *ACT1* mRNA levels.

The data in Figure 4A show that this is indeed the case: if either the K702A or the K702R mutation is introduced into Ire1p-Δtail, the intragenic complementation with full-length Ire1p-K702R is no longer observed. Expression of Ire1p-ΔtailK702A or Ire1p-ΔtailK702R, however, inhibited the low level activity of Ire1p-K702R (Figure 4A, compare the fourth, sixth and eighth columns), presumably due to dominant negative effects exerted by the truncated versions.

These data are explained most easily by a model in which the kinase domain in Ire1p-Δtail is active, and the tailless and full-length Ire1p proteins associate and phosphorylate each other after induction of the UPR. However, an alternative model to explain the inter-allelic complementation must also be considered. In particular, we cannot rule out from the data presented so far that the phenotype of cells bearing full-length Ire1p-K702R is due to increased binding of a factor that negatively regulates the UPR, rather than reduced activity of the Ire1p kinase. Were this the case, the intragenic complementation described above might result from titration of the negative factor by Ire1p-Δtail, revealing almost full kinase activity





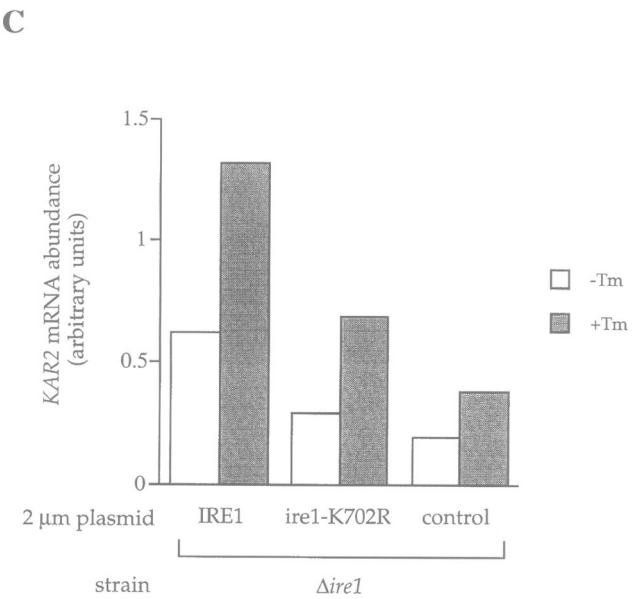


Fig. 4. A wild-type kinase domain in Ire1p-Δtail is required for intragenic complementation with Ire1p-K702R. (**A**) β-Galactosidase assays were carried out on extracts from strain CS309 (which is deleted for the Ire1p coding sequence and bears one copy of the *UPRE-lacZ* reporter gene integrated into its genome) transformed with CEN/ARS plasmids expressing the indicated tailless Ire1p and full-length Ire1p proteins. The Ire1p-Δtail alleles had either a wild-type (w.t.) kinase domain (on plasmid pCS179), or the kinase mutations K702A (pCS180) or K702R (pCS181). Full-length Ire1p-K702R was expressed from plasmid pCS177. The control plasmids (–) are pRS313 (vector backbone of pCS179-181; Sikorski and Hieter, 1989) and YCplac33 (vector backbone of pCS177; Gietz and Sugino, 1988). Yeast cells were grown at room temperature in early log phase in the presence or absence of tunicamycin for 3.5 h before extracts were made. β-Galactosidase assays were carried out as described by Breeden and Nasmyth (1987). (**B**) Overexpression of Ire1p-K702R does not allow induction of a normal UPR. S1 nuclease protection analysis was carried out on RNA isolated from strain CS243 bearing 2 μm plasmids with either wild-type *IRE1* (pCS122), or *ire1*-K702R (pCS178) or made from a related Δ*ire1* strain (CS165) bearing a control 2 μm plasmid (YEplac112; Gietz and Sugino, 1988). Tunicamycin induction followed by S1 nuclease protection analysis was performed as in Cox *et al.* (1993). (**C**) *KAR2* mRNA levels from (B) were quantitated and normalized to *ACT1* mRNA levels. The experiments as shown in (A), (B) and (C) were carried out twice and confirmed additionally by visual color assay on tunicamycin/X-Gal indicator plates (Cox *et al.*, 1993). Results from typical experiments are presented.

of Ire1p-K702R. We consider this explanation highly unlikely, however, because Ire1p-Δtail is not vastly overexpressed but is present in the cells at the same level as Ire1p-K702R (data not shown). Furthermore, it is unlikely that mutation of a single residue in the active site in the Ire1p-Δtail kinase domain would alter the ability of truncated kinase to titrate negative factors away from Ire1p-K702R; yet, such mutations prevent Ire1p-Δtail from complementing Ire1p-K702R (as shown in Figure 4A). Finally, we have measured the UPR in cells overexpressing full-length Ire1p-K702R as their only version of Ire1p. If the activity of Ire1p-K702R were inhibited by binding of a negative regulator when the protein was expressed at wild-type levels, then overexpressing the mutant protein should abolish the inhibition. We found that overexpressing Ire1p-K702R does not restore the UPR to wild-type levels: the response in such cells is <50% of the response observed in cells overexpressing the wild-type Ire1p (Figure 4B and C). We confirmed by immunoblotting that the respective proteins are overexpressed to similar levels in each strain (data not shown). Thus, our data argue against this alternative model and support the first, suggesting strongly that oligomerization and transphosphorylation of Ire1p monomers is required for the UPR.

Truncated Ire1p associates with full-length Ire1p

A dominant negative mutant of Ire1p can be made by truncating Ire1p before the kinase domain ('Ire1p-dom.neg'; see Figure 1). Expression of high levels of Ire1p-dom.neg in cells also expressing normal levels of wild-type Ire1p blocks the UPR after treatment of the cells with tunicamycin (data not shown). Similar dominant negative effects have been reported with other kinase-defective Ire1p mutants (Mori *et al.*, 1993). Given the intragenic complementation results described above, it seemed likely that Ire1p-dom.neg binds to wild-type Ire1p, thereby competing with other full-length Ire1p molecules that would need to associate in order to elicit the UPR. To test this hypothesis, we determined whether hetero-oligomerization of Ire1p and Ire1p-dom.neg could be detected by cross-linking.

A strain expressing both Ire1p-dom.neg and wild-type Ire1p, and control strains expressing either wild-type Ire1p or Ire1p-dom.neg alone, were labeled with [^{35}S]methionine in the presence of tunicamycin. Cells were lysed under non-reducing conditions in the absence of detergent. The reversible, membrane-permeable cross-linker dithiobis-[succinimidylpropionate] (DSP) was then added to the whole cell extracts. After cross-linking, membranes were

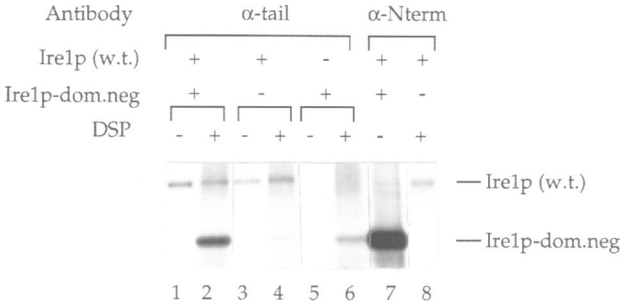


Fig. 5. Truncated Ire1p associates with full-length Ire1p. Strains expressing the indicated Ire1p alleles were grown in galactose (to induce expression of dominant negative Ire1p) in the presence of [^{35}S]methionine and tunicamycin (1 μg/ml) and then extracts were made. Chemical cross-linking was carried out with the reversible cross-linker DSP (200 μg/ml), and Ire1p was immunoprecipitated from the extracts with the indicated antibodies. The cross-linker was reversed before PAGE, and the gel was autoradiographed to visualize the products of the immunoprecipitations. Cells expressing both wild-type (w.t.) and dominant negative (dom.neg) Ire1p were made by transforming wild-type strain JC102 with plasmid pCS114 (bearing Ire1p-dom.neg expressed from the *GAL1/10* promoter). Cells expressing only wild-type Ire1p were JC102 cells transformed with control plasmid pTS210 (bearing the *GAL1/10* promoter) and cells expressing only Ire1p-dom.neg were strain CS309 (*Δire1*) transformed with pCS114. This figure is a composite of non-consecutive lanes of the same exposure of one gel. Because Ire1p-dom.neg is very highly expressed, the amount of immunoprecipitated product loaded in lane 7 is one quarter that loaded in all other lanes. Non-specific bands that migrate to the same position as Ire1p-dom.neg are visible in lanes 4 and 6. Because their intensities are less than one-fifth that of the Ire1p-dom.neg band in lane 2, they do not affect the interpretation of this experiment. Note that, in the presence of DSP, more ^{35}S-labeled Ire1p-dom.neg than wild-type Ire1p immunoprecipitates with the α-tail antibody. This suggests that Ire1p oligomers are larger than dimers. However, the labeling reactions were not necessarily done to steady-state and the possibility that Ire1p-dom.neg has a shorter half-life (and therefore a higher specific activity) than full-length Ire1p cannot be ruled out.

isolated and solubilized in SDS. Immunoprecipitations were carried out with either α-tail antibodies, which bind only to full-length Ire1p but not to Ire1p-dom.neg, or with α-Nterm antibodies, which bind to both versions of Ire1p. The products of the immunoprecipitations were reduced with dithiothreitol (DTT) to reverse the cross-linking and displayed by SDS–PAGE. The results demonstrate that Ire1p-dom.neg can be immunoprecipitated with wild-type Ire1p in a DSP-dependent manner using the α-tail antibody (Figure 5, lanes 1 and 2). This analysis shows that the two proteins are closely associated, supporting our model that oligomers of Ire1p carry out the UPR.

Under the experimental conditions where we detected association of these Ire1p molecules, we observed no change in the amount of truncated Ire1p binding to full-length Ire1p upon induction of the UPR (data not shown). This is probably the result of the extreme overexpression of the truncated Ire1p relative to wild-type protein in the cell (see Figure 5, lane 7), as overexpression of the wild-type Ire1p also induces the UPR (Mori *et al.*, 1993; also, see below). To determine whether the oligomerization state of Ire1p indeed changes with induction of the UPR as proposed, the same cross-linking experiment must be carried out in extracts of cells expressing both truncated and full-length Ire1p at wild-type levels. However, we have been unable to perform this experiment successfully, presumably because of the low natural abundance of Ire1p and because our anti-Ire1p antibodies are low affinity.

Ire1p is phosphorylated

Ire1p has many structural and functional properties in common with other transmembrane receptor kinases. Its tail, for example, is required for signal transduction, and its function requires oligomerization. Oligomerization of transmembrane receptor kinases is thought to lead to activation by *trans*-autophosphorylation. To determine whether this scenario also holds true for Ire1p, we asked whether Ire1p is phosphorylated and, if so, whether phosphorylation increases when the kinase is activated by induction of the UPR. We prepared extracts from wild-type cells that had been labeled with [^{32}P]phosphate either in the presence or absence of tunicamycin. Ire1p was immunoprecipitated with α-Nterm antibodies, the products separated by SDS–PAGE and then visualized by autoradiography. As shown in Figure 6A, Ire1p is a phosphoprotein. Its phosphorylation increased reproducibly 2- to 3-fold with induction of the UPR (lanes 3 and 4).

We next looked at the phosphorylation state of the full-length Ire1p-K702A and Ire1p-K702R mutants in cells grown in the presence of tunicamycin (Figure 6B, lanes 2 and 3). We found that neither protein was phosphorylated under our experimental conditions. This result is consistent with a model in which Ire1p phosphorylates itself. On the basis of these data alone, however, we cannot formally rule out more indirect mechanisms according to which the Ire1p kinase domain is required for phosphorylation of a second protein, itself a kinase or one that activates another kinase, which then in turn phosphorylates Ire1p.

If Ire1p phosphorylates itself, this could occur by an intramolecular mechanism by which the kinase domain phosphorylates groups on the very same molecule or by an intermolecular mechanism by which two Ire1p molecules in spatial proximity phosphorylate each other *in trans*. The interaction between Ire1p-Δtail and Ire1p-K702R described above allowed us to address this issue experimentally. To this end, we determined whether Ire1p-K702R (bearing a compromised kinase domain) is phosphorylated in cells in which Ire1p-Δtail (bearing a wild-type kinase domain) is also present. Yeast cells bearing both versions of the kinase (Ire1p-K702R and Ire1p-Δtail) and strains bearing either version alone (Ire1p-K702R or Ire1p-Δtail) were labeled with [^{32}P]phosphate, and Ire1p was immunoprecipitated as described above. Because the Ire1p-Δtail lacks the C-terminal 133 amino acids, the two forms of Ire1p can be distinguished by their mobilities in SDS–polyacrylamide gels. First, we observed that phosphorylation of Ire1p-Δtail can be detected in cells expressing only that construct (Figure 6C, lane 3). This result provides further evidence that the Ire1p-Δtail kinase domain is active even though Ire1p-Δtail cannot propagate the UPR. Because the intensity of the labeling approaches that found in cells expressing wild-type Ire1p, this result also demonstrates that few, if any, of the Ire1p phosphorylation sites are in the tail. Second, we observed that no phosphorylation is detectable in cells expressing Ire1p-K702R alone (Figure 6C, lane 2); however, in the presence of Ire1p-Δtail, Ire1p-K702R becomes phosphorylated (Figure 6C, lane 1). These results strongly suggest that

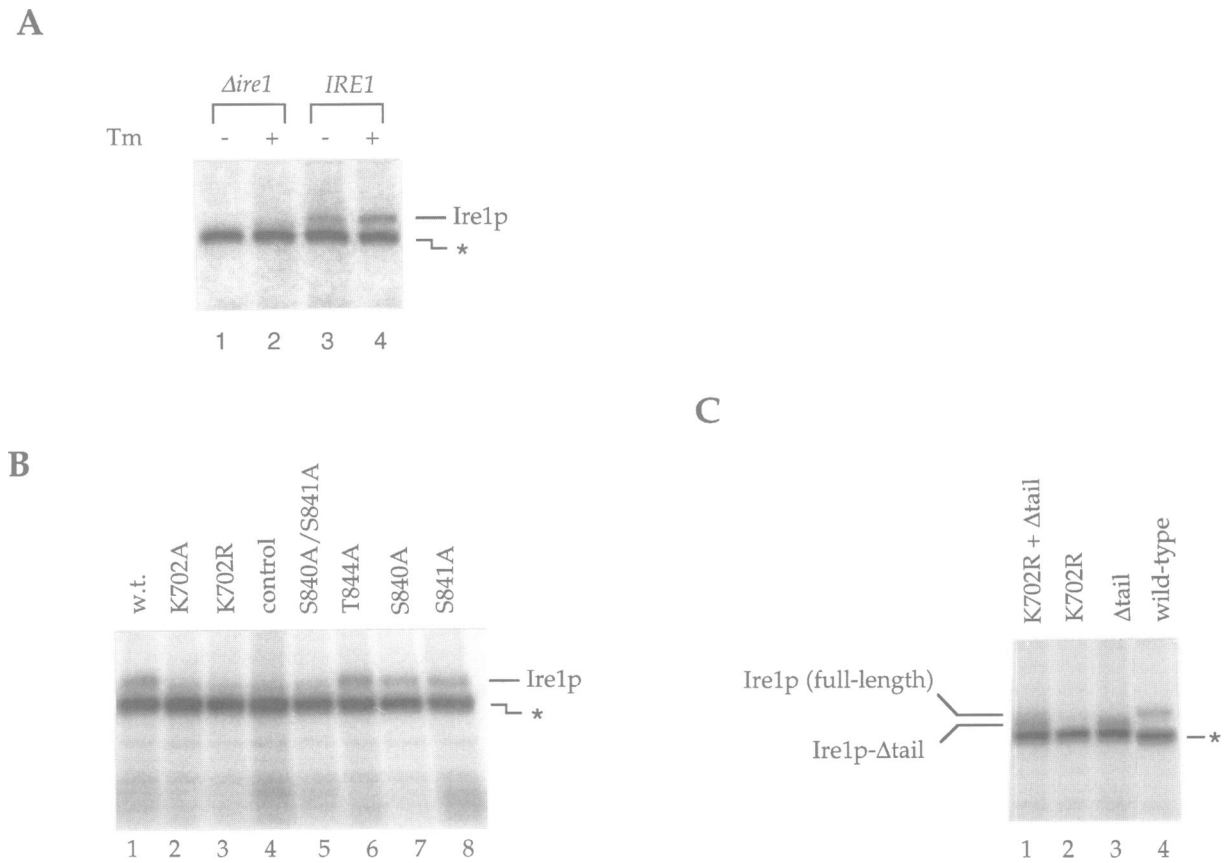


Fig. 6. Phosphorylation of Ire1p. (**A**) Phosphorylation of Ire1p increases with the UPR. Immunoprecipitaion of Ire1p with α-Nterm antibodies from strain CS243 (Δ*ire1*), deleted for the Ire1p coding sequence, and wild-type strain JC102 (*IRE1*). The cells were labeled with [^{32}P]orthophosphate in the presence or absence of tunicamycin (Tm) before being harvested. (**B**) Some Ire1p mutants are not phosphorylated. Immunoprecipitations of extracts from [^{32}P]orthophosphate-labeled cells were carried out as described in (A) except all strains were grown in the presence of tunicamycin. The strains used are all based on strain CS309 (Δ*ire1*) and bear CEN/ARS plasmids expressing either wild-type Ire1p (w.t.; pCS175), no Ire1p (control; YCplac33; Gietz and Sugino, 1988) or an Ire1p mutant (K702A, pCS176; K702R, pCS177; S840A/S841A, pCS171; T844A, pCS172; S840A, pCS185; S841A, pCS187). (**C**) Ire1p is phosphorylated *in trans*. Immunoprecipitations of [^{32}P]orthophosphate-labeled cell lysates were carried out as described in (A) except all strains were grown in the presence of tunicamycin. In lanes 1–3, the cells used were all strain CS309 (Δ*ire1*) transformed with one or two CEN/ARS plasmids bearing the indicated *IRE1* alleles (*ire1*-Δtail on pCS179, and/or full-length *ire1*-K702R on pCS177); cells expressing only one *IRE1* allele also carried one of two control plasmids (pRS313; Sikorski and Hieter, 1989; or YCplac33; Gietz and Sugino, 1988). The wild-type strain in lane 4 is JC102 transformed with a control plasmid (YCplac22; Gietz and Sugino, 1988). The results shown in (A), (B) and (C) are representative of experiments that were repeated at least once for each strain. A highly phosphorylated background band that migrates faster than Ire1p is visible in all lanes (*). Its intensity did not change with the induction of the UPR. Immunoprecipitations were carried out on cell extracts which were made from equivalent numbers of yeast cells and which were shown subsequently to have approximately equal protein concentrations.

phosphorylation of Ire1p occurs, at least in part, *in trans*, i.e. is carried out by neighboring Ire1p molecules.

Ire1p is phosphorylated primarily on serine residues

Sequence comparisons with other kinases suggest that Ire1p is a serine/threonine kinase. To determine its specificity, we carried out phosphoamino acid analysis on Ire1p isolated from cells labeled with [^{32}P]phosphate (see Materials and methods). Only phosphoserine could be detected in Ire1p from cells expressing wild-type levels of the protein (strain JC102), and the same result was obtained whether Ire1p was isolated from cells grown in the presence or absence of tunicamycin (Figure 7A and B). Because we could obtain only very small amounts of phosphorylated Ire1p from JC102 cells, we also carried out phosphoamino acid analysis on Ire1p isolated from cells overexpressing the protein. Using this much more abundant source of Ire1p, we confirmed that serine is the predominant phosphoamino acid in Ire1p, but we detected a small of amount of phosphothreonine as well (Figure 7C).

It should be noted that cells overexpressing Ire1p do not behave exactly like wild-type cells. As has been reported previously (Mori *et al.*, 1993) and as is shown in Figure 4B and C, in the absence of tunicamycin, cells expressing high levels of wild-type Ire1p constitutively induce the unfolded protein response to a level that is ~50% of that in response to tunicamycin. Cells overexpressing Ire1p also have enlarged vacuoles and seem to have extra ER membranes (C.E.Shamu, J.Mulholland and D.Botstein, unpublished observations). Thus, they are probably not physiologically identical to cells expressing wild-type levels of the protein. Results obtained from cells overexpressing Ire1p must thus be interpreted with caution. It therefore remains to be established whether the small amount of phosphothreonine detected on Ire1p isolated from the overproducing strain is physiologically relevant.

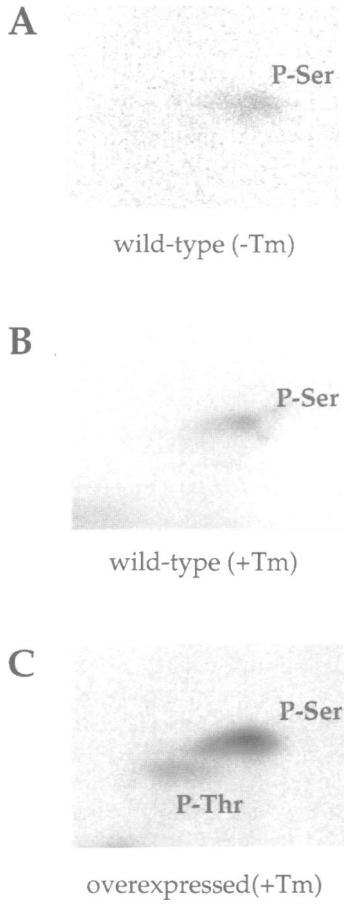


Fig. 7. Ire1p is phosphorylated primarily on serine. Phosphoamino acid analysis was carried out on phosphorylated Ire1p immunoprecipitated from strains expressing wild-type levels of Ire1p (JC102), (**A**) and (**B**), or overexpressing the protein from a 2 μm plasmid (pCS122 in strain CS243), (**C**). As indicated, strains were grown in either the presence or absence of tunicamycin (Tm).

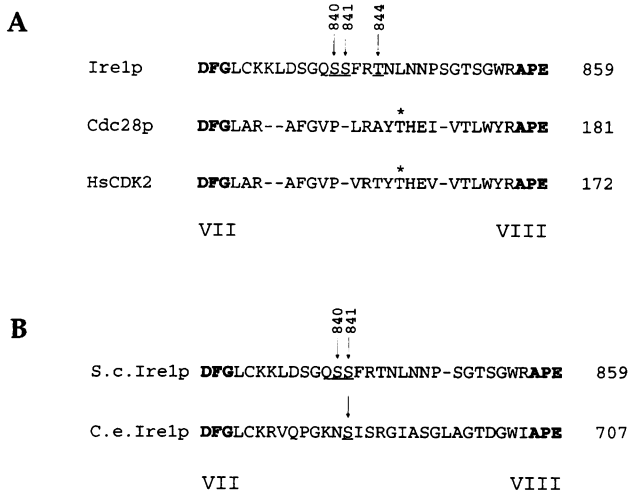


Fig. 8. The VII–VIII loop of Ire1p. (**A**) Possible activating phosphorylation sites in Ire1p. A sequence comparison of the kinase domain VII–VIII loops from *S.cerevisiae* Ire1p, *S.cerevisiae* Cdc28p and human CDK2. Known activating phosphorylation sites are starred and candidate activating sites in Ire1p (S840, S841 and T844) are indicated with arrows. (**B**) A comparison of the kinase domain VII–VIII loops in *S.cerevisiae* Ire1p and in a sequence homolog of Ire1p from *C.elegans* (GenBank accession no. Z48045; Wilson *et al.*, 1994).

On which residue(s) is Ire1p phosphorylated?

To begin to study the role of phosphorylation in Ire1p function, we wished to identify which of its residues are phosphorylated. The CDK kinases, which lie closest to Irep on the kinase phylogenetic tree (Hanks and Hunter, 1995), and many transmembrane tyrosine kinases have activating phosphorylation sites on a protein loop that stretches between conserved kinase domains VII and VIII (Figure 8A; for example, see Hubbard *et al.*, 1994; Morgan and De Bondt, 1994, and references therein). Between domains VII and VIII, Ire1p has three serine and threonine residues that, based on their location in the loop, seem good candidates for activating phosphorylation sites: serine 840 (S840), serine 841 (S841) and threonine 844 (T844). We tested the role of these putative phosphorylation sites by mutating them, singly or in combination, to alanine and then examining the phenotypes of cells bearing the different Ire1p mutations.

We first tested the double mutant, Ire1p-S840A/S841A, and Ire1p-T844A. Interestingly, as measured by S1 nuclease protection assays, the S840A/S841A mutation virtually eliminated the UPR whereas the T844A mutation only reduced the response by ~30% relative to wild-type (Figure 9A, lanes 5–8). These phenotypes correlated with the ability of the mutants to grow on plates lacking inositol: Ire1p-S840A/S841A mutants barely grew, while Ire1p-T844A mutants grew as well as wild-type cells (data not shown). When we examined the phosphorylation state of the mutant Ire1p proteins, we found that Ire1p-S840A/S841A was not phosphorylated while Ire1p-T844A was (Figure 6B, lanes 5 and 6). By immunoprecipitation of [^{35}S]methionine-labeled Ire1p, we confirmed that the expression levels of the Ire1p mutants in test strains were the same as those of wild-type Ire1p in control strains (data not shown). Given the phosphoamino acid data presented above, we thought it likely that S840 and/or S841 might represent the activating phosphorylation sites in Ire1p. This hypothesis was supported by sequence comparisons of *S.cerevisiae* Ire1p and a sequence homolog of Ire1p in *Caenorhabditis elegans* which was entered into the protein sequence database recently (Wilson *et al.*, 1994). A serine in the vicinity of amino acid 840 or 841 is conserved whereas there is no threonine nearby in the *C.elegans* gene (Figure 8B).

To test this hypothesis, we made two additional Ire1p mutants, Ire1p-S840A and Ire1p-S841A, in which the serines were changed separately to alanine. Again, both mutants are expressed in test strains at the same levels as wild-type Ire1p in control strains. When we examined the UPR in these mutants, we found that, although S841A has a stronger effect than S840A, neither mutation alone affects the response as strongly as does the double mutant (Figure 9A, lanes 5, 6, 9, 10, 11 and 12). Furthermore, both Ire1p-S840A and Ire1p-S841A are phosphorylated *in vivo*, although to reproducibly lower levels than the wild-type protein (Figure 6B, lanes 7 and 8) and the Ire1p-T844A mutant. Thus, it is possible that neither serine is the activating phosphorylation site on Ire1p and that the severe phenotype of the S840A/S841A double mutant is a result of structural problems in the kinase domain that simply destroy its activity. However, a more interesting interpretation of these results is that the two serines form

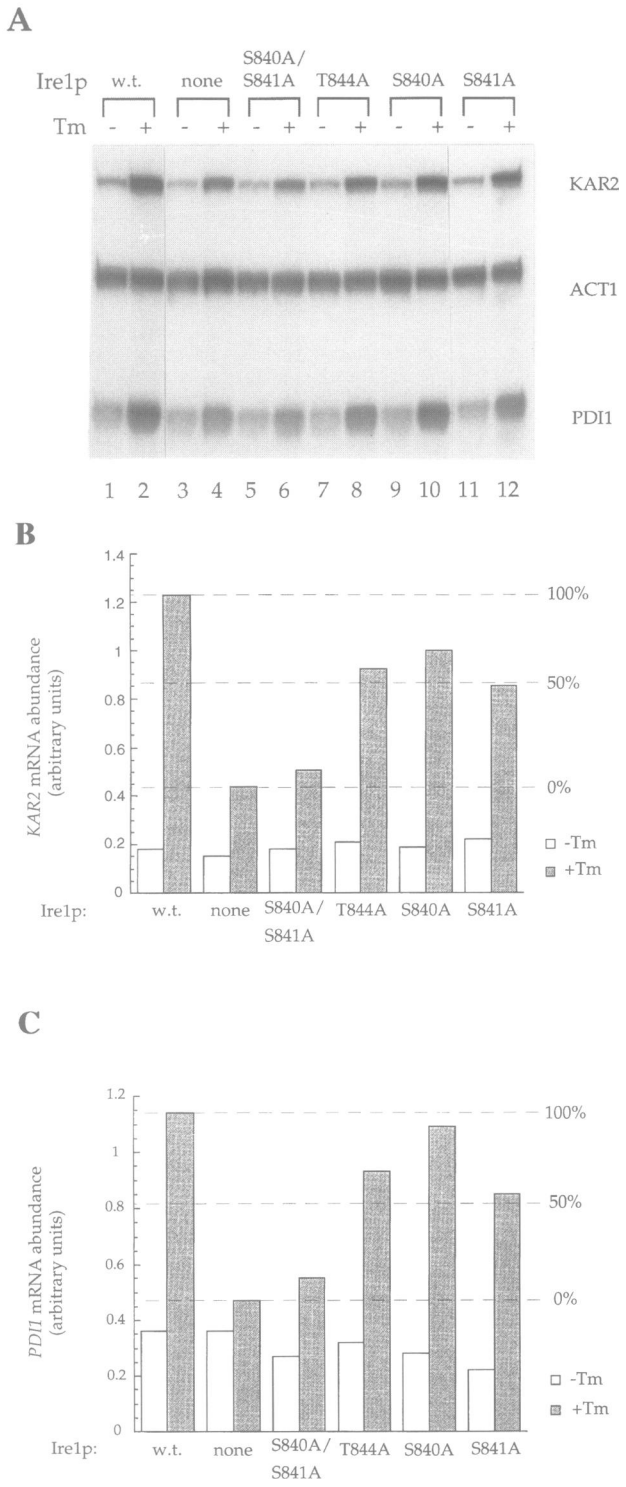


Fig. 9. Mutation of putative Ire1p activating phosphorylation sites affects the UPR. (**A**) S1 nuclease protection analysis of RNA isolated from strain CS309 (*Δire1*) transformed with CEN/ARS plasmids expressing the indicated Ire1p alleles [wild-type, pCS175; control, YCplac33; (Gietz and Sugino, 1988); S840A/S841A, pCS171; T844A, pCS172; S840A, pCS185; or S841A, pCS187]. Tunicamycin induction followed by S1 nuclease protection analysis was performed as in Cox *et al.* (1993). (**B**) *KAR2* mRNA levels from (A) were quantitated and normalized to *ACT1* mRNA levels. (**C**) *PDI1* mRNA levels from (A) were quantitated and normalized to *ACT1* mRNA levels. The experiment as shown was carried out twice and confirmed additionally by visual color assay on tunicamycin/X-Gal indicator plates (Cox *et al.*, 1993). Results from a typical experiment are presented.

partially redundant activating phosphorylation sites, with S841 perhaps being the more important of the two, that can compensate for each other when only one is mutated because they lie so close together. Because the activity of Ire1p is probably regulated by its rates of phosphorylation and dephosphorylation, two partially redundant sites could dramatically increase the dynamic range of the response.

Discussion

Here we show that activation of Ire1p and transmission of the unfolded protein signal occurs by mechanisms similar to those found in many higher eukaryotic plasma membrane receptor kinases. In particular, we have presented molecular genetic and direct biochemical evidence that Ire1p oligomerizes and is activated by *trans*-autophosphorylation. The Ire1p tail domain is required for the UPR, possibly to bind additional components in the pathway that then become activated and transmit the signal to the transcription machinery in the nucleus.

The intragenic complementation between the Ire1p-K702R and Ire1p-Δtail alleles (Figures 3C and 4A) and the phosphorylation studies on cells bearing these proteins (Figure 6C) can be explained most easily by the model diagrammed in Figure 10A. The data suggest that the Ire1p-Δtail contains an active kinase domain but cannot signal. Oligomerization of Ire1p-Δtail and full-length Ire1p-K702R allows for cross-talk between the two and compensation for the defect in each. The full-length Ire1p-K702A mutant, whose kinase is probably inactive (or at the least considerably less active than that of Ire1p-K702R), cannot 'talk' to the tailless mutant, and thus complementation does not occur. Signaling would be initiated by oligomerization. In the absence of phosphorylation on the activation loop, both interacting kinases may display low levels of activity that are sufficient to add a phosphate group occasionally to the activation loop of the other. Once this event occurs, it would trigger a positive feedback loop, as the activated kinase would in turn activate its partner (Figure 10A, '1'). Thus activated, the kinase would phosphorylate either the tail of its partner or any protein that may be held in proximity because of interactions with the Ire1p tail (Figure 10A, '2', and Figure 10B) as the next step in the propagation of the signal.

We have demonstrated that the phosphorylation of Ire1p increases with the induction of the unfolded protein response. Just as many other transmembrane receptor kinases oligomerize and autophosphorylate in response to ligand binding, it is likely that the increase in Ire1p phosphorylation is a result of an increase in Ire1p oligomerization as unfolded proteins accumulate in the ER. The transcomplementation of two individually impaired Ire1p mutants strongly suggests that Ire1p exists as an oligomer once the UPR has been induced. We have also confirmed biochemically that oligomerization can occur: an overexpressed truncated version of Ire1p (Ire1p-dom. neg) could be cross-linked to full-length wild-type Ire1p (Figure 5). Interestingly, under the conditions where we see association of Ire1p-dom.neg with wild-type Ire1p, stoichiometrically more ^{35}S-labeled truncated Ire1p than labeled full-length Ire1p immunoprecipitates with an antibody that binds only to the full-length protein. This

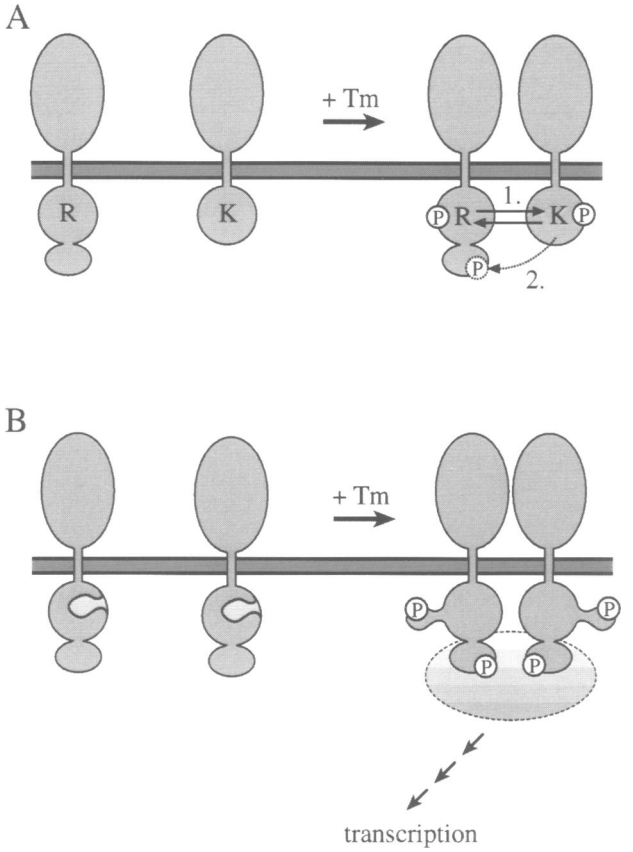


Fig. 10. Model for Ire1p activation. (**A**) A model for the intragenic complementation between *ire1* alleles. When the UPR is induced in cells bearing both Ire1p-Δtail and Ire1p-K702R (+Tm), oligomers of the two mutant proteins form and each kinase phosphorylates the other on kinase-activating phosphorylation sites (1). Additionally, truncated Ire1p (that has a wild-type kinase domain) phosphorylates the tail of Ire1p-K702R and/or a protein that binds to the tail (2). This second phosphorylation event would be required for transmission of the unfolded protein signal. (**B**) A model for the role of Ire1p in the UPR pathway. Induction of the UPR (+Tm) causes Ire1p monomers to oligomerize and phosphorylate each other. Phosphorylation of the kinase activation loop removes steric hindrances of substrate entry into the active site. Phosphorylation of the Ire1p C-terminal tail or of other, as yet unidentified, proteins that bind to the tail, allows transmission of the unfolded protein signal toward the nucleus and induction of transcription of the genes encoding ER resident proteins.

observation suggests that Ire1p oligomers are larger than dimers.

The study of other transmembrane receptor kinases suggested that phosphorylation of Ire1p might correlate with the activity of the UPR and, given the partial activity of Ire1p-K702R, we had expected to detect some phosphorylation of the mutant protein. We found instead that Ire1p-K702R is not phosphorylated detectably. Thus, phosphorylation of Ire1p may correlate more closely with the kinase activity of Ire1p than with the UPR as measured by induction of *KAR2* transcription, which is probably many steps removed. It seems likely that Ire1p-K702R has a very weak kinase activity, whereas Ire1p-K702A may have virtually none. The magnitude of the UPR in cells bearing Ire1p-K702R would result from an amplification of the weak kinase activity in later steps in the pathway.

It is likely that kinase-activating phosphorylation sites in Ire1p lie in the loop that connects conserved domains VII and VIII of the kinase (Hanks and Hunter, 1995). The crystal structures of human CDK2, of the MAP kinase ERK2 and of the tyrosine kinase domain of the human insulin receptor have shown that, in the absence of the activating phosphorylation, the VII–VIII loop physically blocks access to the active site of each kinase, thereby inhibiting substrate binding (De Bondt *et al.*, 1993; Hubbard *et al.*, 1994; Zhang *et al.*, 1994). It is thought that phosphorylation on the loop activates the kinase at least in part by removing the loop from the substrate binding site by allowing it to bind stably to positively charged residues located elsewhere in the protein. Our data suggest that S840 and S841 are both activating phosphorylation sites in Ire1p that are phosphorylated in response to the accumulation of unfolded proteins in the ER. The presence of more than one phosphorylation site in the VII–VIII loop is not unprecedented, as several kinases, such as insulin receptor and Mek1, also have multiple activating phosphorylation sites in this loop (Huang and Erikson, 1994; White *et al.*, 1988). In fact, in the insulin receptor, two of the phosphorylated residues lie side by side, and mutation of only one of the two residues diminishes but does not abolish the ability of the receptor to autophosphorylate (Ellis *et al.*, 1986). However, we cannot rule out the possibility that other serine and/or threonine residues in the Ire1p VII–VIII loop (e.g. S837, T844, S850 or T852) may be activating phosphorylation sites in addition to or instead of S840 and S841.

We have presented here data that support the model for activation of Ire1p diagrammed in Figure 10B. When few unfolded proteins accumulate in the ER, Ire1p exists mostly as a monomer that has a low, basal level of kinase activity. As the concentration of unfolded proteins increases, Ire1p oligomerizes and the Ire1p molecules phosphorylate each other. Oligomerization of Ire1p might be triggered by its binding directly to unfolded proteins or by other components such as BiP (Shamu *et al.*, 1994). Phosphorylation probably occurs on activating phosphorylation sites in the kinase domain that increase kinase activity. Phosphorylation may also occur on sites in the Ire1p tail domain or on proteins that bind to the Ire1p tail. How the activated Ire1p receptor transmits the unfolded protein signal to the nucleus remains to be determined.

Materials and methods

Strains and plasmids

The strains used in this study are listed in Table I. The *ire1* disruption in strain CS236 is the same as in CS165. The *ire1* disruptions in strains CS243 and CS309 remove only the Ire1p coding sequence and were created by homologous recombination using PCR-amplified fragments of *IRE1* ligated into plasmid pRS306 (URA^+, for CS243) or plasmid pRS304 (TRP^+, for CS309; Sikorski and Hieter, 1989). Sequences truncating Ire1p at amino acid 982 (*ire1*-Δtail, strain CS240) were introduced into the *IRE1* gene by homologous recombination (Sikorski and Hieter, 1989). The integrating vector used was YIplac204 (TRP^+; Gietz and Sugino, 1988) and the mutant *ire1* insert was created by PCR. The resulting *ire1* gene has the *ACT1* transcriptional terminator (bases 1666–1760; Ng and Abelson, 1980) in place of the *IRE1* terminator.

Plasmid pCS114 expresses a mutant Ire1p that is truncated at amino acid 674 and contains the amino acids AMA added just before the stop codon. In pCS114, truncated *ire1* is cloned into the URA^+ CEN/ARS plasmid pTS210 (gift of Tim Stearns, Stanford University), which places it under control of the *GAL1/10* promoter and the *ACT1* transcriptional terminator. Site-directed mutagenesis of Ire1p was carried out by standard methods (Kunkel *et al.*, 1987); all mutated fragments of *IRE1* were

Table I. Yeast strains

Strain	Genotype	Source/reference
JC102	*leu2-3,-112, his3-11,-15, trp1-1, ura3-1, ade2-1, can1-100 MATa, leu2-3,-112::LEU⁺ UPRE-lacZ*, MET⁺	this study
CS165	As JC102, except *his3-11,-15::HIS⁺ UPRE-lacZ, ire1::URA3* (only 2/3 of Ire1p coding sequence deleted), met⁻	Cox *et al.* (1993)
CS236	As JC102, except *ire1::URA3* (only 2/3 of Ire1p this study coding sequence deleted)	this study
CS240	As JC102, except *ire1:: TRP1 ire1-Δtail*	this study
CS243	As JC102, except *ire1::URA3* pRS306 (entire Ire1p coding sequence deleted; Sikorsi and Hieter, 1989)	this study
CS309	As JC102, except *ire1::TRP1* pRS304 (entire Ire1p coding this study sequence deleted; Sikorsi and Hieter, 1989)	this study

sequenced to confirm that only the desired mutations had been introduced into the gene. All plasmids expressing full-length Ire1p bear the *Xho*I–*Hin*dIII fragment of the *IRE1* gene (Cox *et al.*, 1993). Plasmid pCS122 bears wild-type *IRE1* in the high copy vector backbone YEplac112 (Gietz and Sugino, 1988). pCS178 is identical to pCS122 except the K702R mutation has been introduced into *ire1*. pCS175 bears wild-type *IRE1* in the CEN/ARS vector YCplac33 (Gietz and Sugino, 1988). Plasmids pCS171 (S840A/S841A), pCS172 (T844A), pCS176 (K702A), pCS177(K702R), pCS185 (S840A) and pCS187(S841A) are identical to pCS175 except the indicated mutations were introduced into *ire1*. Plasmid pCS179, expressing Ire1p-Δtail (with a wild-type kinase domain) bears *IRE1* truncated exactly as described for yeast strain CS240. Expression of the *ire1*-Δtail gene is driven by the *IRE1* promoter (starting at the upstream *Xho*I site, Cox *et al.*, 1993) and the *ACT1* transcription terminator is present at the 3′ end of the gene. The vector backbone of pCS179 is pRS313 (HIS⁺; Sikorski and Hieter, 1989). pCS180 (ΔtailK702A) and pCS181 (ΔtailK702R) are identical to pCS179 except for the indicated point mutations.

Antibodies

α-Ire1Nterm and α-Ire1tail antibodies were raised against GST/Ire1p fusion proteins (Smith *et al.*, 1986; Smith and Johnson, 1988). Plasmids encoding the GST–Ire1p fusions were made using Vent polymerase (New England Biolabs, Beverly, MA) and PCR to amplify the appropriate fragment of the *IRE1* gene [Ire1Nterm, bases 413–1918; Ire1tail, bases 3281–3700; (Nikawa and Yamashita, 1992)] and the fragment was cloned into the pGEX4T-2 (Ire1Nterm) or pGEX2T GST (Ire1tail) expression vectors (Pharmacia, Piscataway, NJ). The DNA sequence of the GST–Ire1p junctions was determined to be correct in plasmids derived from two independent PCR reactions for each construct. Because Vent polymerase has relatively high fidelity, the rest of the each construct was not sequenced further. *Escherichia coli* transformed with the GST–Ire1 plasmids produced GST fusions of the expected molecular weight (~83 kDa for Ire1Nterm and 43 kDa for Ire1tail). The GST–Ire1 fusion proteins were not very soluble in buffers containing 1% Triton X-100. Thus, they were isolated from bacteria in inclusion bodies, partially purified by PAGE and eluted from gel slices with the Elutrap electroelution system (Schleicher and Schuell, Keene, NH) before being injected into rabbits.

In vivo labeling and immunoprecipitation

Yeast cells, grown at room temperature in medium containing appropriate supplements and lacking methionine, were labeled with [^{35}S]methionine and non-native immunoprecipitations (IPs; done after SDS denaturation) were carried out essentially as described by Hann and Walter (1991). The cells were labeled for a total of 1.5 h with 100 μCi of [^{35}S]methionine (Pro-mix ^{35}S cell labeling mix, Amersham Life Sciences, Arlington Heights, IL) per OD_{600} unit of cells. If added, tunicamycin (1 μg/ml) was present for the last hour (Figure 2A) of labeling. Cells were lysed in the presence of 10% trichloroacetic acid (TCA); TCA pellets were resuspended in 100 mM Tris with 1% SDS and 3 mM DTT and heated to 65°C for 10 min. Cell extracts were then diluted so that each IP reaction was done with the extract equivalent of 1 OD_{600} of cells in 600 μl of 20 mM Tris pH 7.5, 150 mM NaCl, 0.2% SDS, 1% Triton X-100. Ten μl of α-Nterm or α-tail polyclonal serum was added to each IP and the α-Ire1p antibodies were collected with 50 μl of a 25% suspension of heat-inactivated *Staphylococcus aureus* (Pansorbin cells, Cal Biochem, San Diego, CA) per IP. *S.aureus* is much more efficient in collecting α-Ire1p antibodies than protein A–Sepharose, suggesting that the α-Ire1p antibodies have low affinity for Ire1p. Under the experimental conditions in Figure 2A, >85% of Ire1p in each [^{35}S]-methioine-labeled lysate was precipitated. The addition of phosphatase inhibitors to extracts diminished this efficiency somewhat (see below).

Yeast cells grown in low phosphate medium (O'Connell and Baker, 1992) were labeled with [^{32}P]orthophosphate (NEX-053, DuPont, Wilmington, DE) at room temperature. Cells grown overnight in the appropriate high phosphate medium to log phase were washed into low phosphate medium and grown for 4 h. Five OD_{600} units per strain were then diluted to 0.77 OD_{600}/ml in low phosphate medium and [^{32}P]orthophosphate was added to a concentration of 100 μCi/ml. Cells were incubated, with shaking, at room temperature for 15 min before tunicamycin (to a final concentration of 1 μg/ml) was added (or not) and the cultures were grown for an additional 30 min before harvesting. Extracts and non-native immunoprecipitations were carried exactly as described for [^{35}S]methionine-labeled cells, except phosphatase inhibitors (10 mM NaF, 80 mM β-glycerol phosphate, 1 mM sodium vanadate and 10 μm calyculin A) were present in all buffers and 10 mM sodium phosphate pH 7.5 was added to the immunoprecipitation and to the first wash of the *S.aureus* pellet. Each IP reaction was done with the extract equivalent of 1 OD_{600} of cells. The protein concentration of each cell extract was also determined using the Micro BCA protein assay (Pierce, Rockford, IL) to confirm that all extracts were prepared from approximately the same numbers of cells. Under these immunoprecipitation conditions, >65% of Ire1p in each lysate was precipitated.

Cross-linking

Chemical cross-linking was carried out on extracts of cells that had been labeled, as described above, with [^{35}S]methionine. Tunicamycin was present for the last 30 min of labeling. Extracts were made by bead beating cells in HLB (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 200 mM sorbital) plus protease inhibitors (10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride) and then spinning the lysate at 1000 r.p.m. (Beckman JS13 rotor) for 4 min to remove unbroken cells. The reversible cross-linker DSP [Pierce, Rockford, IL; dissolved in dimethylsulfoxide (DMSO)] or an equal volume of DMSO was added to the extract equivalent of 10 OD_{600} of cells in each cross-linking reaction. Reactions proceeded for 20 min at 4°C and were quenched by the addition of an equal volume of 200 mM ammonium acetate. Membranes and insoluble material were pelleted in a 12 min centrifugation at 35 000 r.p.m. (Beckman TL100.3). The pellet was resuspended in HLB plus protease inhibitors and then solubilized by the addition of SDS to 1%. Non-native IPs (using an extract equivalent of 5 OD_{600} of cells per IP) were then carried out exactly as described above.

Phosphoamino acid analysis

Two-dimensional phosphoamino acid analysis was carried out on Ire1p that had been immunoprecipitated from extracts of cells labeled with [^{32}P]orthophosphate and eluted from gel slices. The phosphoamino acid analysis protocol followed is the same as that described by Boyle *et al.* (1991). To analyze Ire1p from JC102 cells, the appropriate slices from three separate lanes of an acrylamide gel (each loaded with the products from one IP reaction described above for [^{32}P]orthophosphate-labeled cells) were combined before eluting the protein and, after hydrolysis, all of each sample was loaded on a TLC plate for analysis. For Ire1p from Ire1p-overexpressing cells, protein was eluted from one band from one lane of the gel (loaded with the products of one IP reaction) and one-half of the resulting hydrolyzed sample was loaded on the TLC plate.

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